



# PALM TISSUE AND ORGAN CULTURE PROTOCOLS

केन्द्रीय रोपण फसल अनुसंधान संस्थान

(भारतीय कृषि अनुसंधान परिषद)

कासरगोड - 671 124, केरल, भारत



CENTRAL PLANTATION CROPS RESEARCH INSTITUTE

(Indian Council of Agricultural Research)

Kasaragod - 671 124, Kerala, India



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### Front Cover

1. Non-destructive sampling of arecanut inflorescence from Hirehalli dwarf variety.
2. Embryogenic calli from inflorescence explants of arecanut after 6 months
3. Plantlet regeneration from inflorescence explants of arecanut
4. Established arecanut plantlets in polybags
5. Plantlet regeneration from arecanut inflorescence explants of Hirehalli Dwarf.



### Back Cover

1. Embryogenic calli and somatic embryos from plumular explants of coconut
2. Germination of coconut somatic embryo from plumular tissue
3. Plantlet from plumular explant ready for pot transfer
4. Nut set in of CGD palm –derived through plumular culture

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## FOREWORD

Plant tissue culture programmes were initiated at Central Plantation Crops Research Institute during 1970s and a number of protocols have been evolved during the past three and a half decades. Though palm tissue culture programmes were started at many institutions in the country, only at this Institute the experiments are continuing in a systematic manner. Some of the protocols developed at CPCRI have received international attention, especially the coconut zygotic embryo culture protocol. It is being recommended by Bioversity International (formerly the IPGRI) for coconut germplasm exchange. The CPCRI protocol was successfully employed in eight germplasm expeditions and plantlets retrieved were field planted at International Coconut Gene Bank for South Asia (ICG-SA), Kidu. The Indian Council of Agricultural Research recognized these efforts by conferring the Best Team Research Award during 2002.

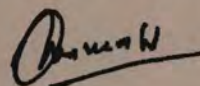
Not many laboratories in the world are engaged in coconut tissue culture. The progress made by CPCRI laboratory in coconut tissue culture is comparable with that of other international laboratories. The coconut plumule culture is found to be the best alternative and palms derived through this method have already started yielding. Few plants from inflorescence tissues have also been regenerated.

Clonal propagation of arecanut has become a reality and the protocol has been standardized with inflorescence explants. Large number of plantlets, especially from the YLD resistant palms, could be produced. Embryo culture of arecanut has also been standardized.

Other palm tissue culture protocols developed at this centre include leaf tissue culture of juvenile oil palm, embryo rescue in *pisifera* palms etc. This technical bulletin describes the steps involved in various tissue/organ culture protocols evolved at CPCRI and is expected to serve as a reference guide to research personnel involved in tissue culture of plantation crops.

March, 2008

(George V. Thomas)



Director

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## 1. INTRODUCTION

The long juvenile phase, heterozygous and out crossing nature, and requirement of large area for experimentation make it difficult to achieve genetic improvement in palms a difficult task. The low reproductive rate further restricts large-scale cultivation of improved varieties. This situation has compelled farmers to go for indiscriminate planting of seedlings and this has resulted in low productivity in large extends of area under palms. Standardization of commercially viable tissue culture protocols in palms has become the need of the hour to meet the requirement of quality planting material.

The medium used for periodic subculturing, culture conditions, selection of explants, regulation of hormones and acclimatization procedures etc. are the pivotal elements of a tissue/organ culture protocol. The success of the protocol for repetitive and commercial application depends upon how correctly the

aforesaid components are followed. To meet this requirement the key elements of the protocols developed at CPCRI for tissue/organ culture in coconut, arecanut, and oil palm are listed in this publication.

Coconut tissue culture using plumule tissues, arecanut tissue culture using inflorescence explants and oil palm tissue culture are dealt in Chapter 2.

The CPCRI protocol of coconut zygotic embryo culture, arecanut embryo culture and embryo rescue in *pisifera* are discussed in Chapter 3. A section to describe the germplasm expedition using the CPCRI protocol of coconut embryo culture is also included in this Chapter.

The Y3 and MS media are the commonly used basal medium for the aforesaid protocols. Annexure 1 and 2 provide the nutrient combinations of Y3 and MS media.

## 2. TISSUE CULTURE

### 2.1. Coconut (*Cocos nucifera* L.) plumular tissue culture

#### 2.1.1. Extraction of explants:

- Scoop out zygotic embryos with a portion of endosperm using a cork borer from dehusked and split opened mature coconuts.(fig.30)
- Extract embryo from the endosperm with the help of a small knife/scalpel.
- Surface sterilize the embryos with 50 % chlorine water or 20% sodium hypochlorite for 20 minutes and subsequent wash the embryos three to four times with sterilized water to remove the traces of chlorine.

#### 2.1.2. Conditioning of the embryos

- Inoculate surface sterilized embryos into Y3 medium (Annexure I) containing sucrose (3%), charcoal (0.1 %) and agar (0.55 %).
- Adjust pH of the medium to 5.7 to 5.8 before autoclaving.
- Incubate the cultures in dark for a month at  $27 \pm 2$  °C.

#### 2.1.3. Preparation of explants

- After a month of incubation of embryos in germination medium, the embryo enlarges. Slice out the plumular region with the help of sharp scalpel. Each plumular region/embryo can give about 4-5 slices (Fig. 1)
- Inoculate these explants into same basal medium supplemented with 2,4-D (16.5

mg/l) with TDZ (1 mg/l) and incubate in dark for callus induction (Fig. 2).

#### 2.1.4. Induction of somatic embryos and meristemoids

- Sub culture the explants to same basal medium supplemented with 2,4-D (8.25 mg/l) with BA (1 mg/l), TDZ (1 mg/l) and putrescine (1 mM)
- Gradually reduce 2, 4-D concentration from 8.25 mg/l to 4 mg/l, then to 2 mg/ at each monthly subculture. Transfer explants finally to medium devoid of 2,4-D.



Fig. 1. Plumular sections



Fig. 2. Nodular calli from plumular tissue



### 2.1.5. Plantlet regeneration

- Observe somatic embryogenesis with an incubation period of 16 weeks (Fig. 3 and 4).
- Transfer germinated embryos (with two leaves and primary root, almost four months after inoculation) to liquid rooting medium (Fig. 5).
- Subculture on the same medium once every 4-5 weeks. Transfer to wide-mouth and longer tubes whenever necessary (Fig. 6).
- Select plantlets with well-developed roots and shoot system (3-4 leaves) for hardening
- Pre-treat the plantlets with Carbendazim (1g/l) and IBA (1000 ppm) for 1 hour each and transfer to the pots.
- Potting mixture consists of sterilized soil: sand: coir dust (1:1:1).



Fig. 3. Germinating somatic embryos



Fig. 4. Germinating somatic embryos



Fig. 5. Meristemoid development



Fig. 6. Germination of somatic embryo derived plantlet



### 2.1.6. Acclimatization

- Cover the plantlets with polyethylene bags for 2-3 weeks and keep them indoors at room temperature with artificial light.
- Supply macro Eeuwen's Y3 (Annexure I) solution once every 15 days.
- Irrigate to keep potting mixture moist.
- After three weeks, harden the plantlets by gradually perforating the polyethylene bags and remove the bags at night for two weeks.
- After two weeks, remove the polythene bags completely and keep plantlets indoors for one week (Fig. 7).
- Transfer plantlets to bigger pots and keep them in net house with 50% shade.
- 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 about 5-6 months.
- Irrigate regularly and apply recommended dose of fertilizer whenever necessary.
- After 4-5 months, plantlets can be transferred to the field (Fig. 8 and 9)



Fig. 7. Well established plantlets in pot



Fig. 8. Field establishment



Fig. 9. Flowering after 36 months of field planting (CGD)



### 2.1.7. Practical Utility

- This protocol could be applied for rapid multiplication of proven hybrids of coconut such as CGD X WCT and MGD X WCT and dwarfs COD (tender nut variety)
- *In vitro* conservation and genetic transformation studies

### 2.1.8. Publications

Devakumar, K., Anitha Karun, Radha, E. and Parthasarathy, V.A. (1999) Morphogenic response of plumule of coconut to 2,4-D and Cytokinins (Abstract No. IV-12). *National Seminar in Plant Physiology and Biochemistry*. CPCRI Kasaragod, 3 rd to 5 th February, 2000.

Rajesh, M.K., Radha, E., Sajini, K.K., Anitha Karun and Parthasarathy, V.A. (2005) Plant regeneration through organogenesis and somatic embryogenesis from plumular explants of coconut. *Journal of Plantation Crops* 33: 9-17.

Besides increasing the multiplicative rate of coconut to many folds, the plumule culture will be handy while initiating transformation studies as the culturing period is considerably less when compared with other explants. It will be ideal for generating large number of planting material from promising exotic collections or from rare genotypes and also the proven hybrids. Further when a genotype is cryopreserved in the form of embryos, its regeneration in large numbers could also be possible by means of plumule culture.

## 2.2. Arecanut (*Areca catechu* L.) tissue culture (explant: inflorescence)

### 2.2.1. Extraction of explants

- Sample inflorescence (spadix length of 10-25 cm) from adult palms in the field non-destructively (Fig.10) by removing the outer leafsheaths. Care should be taken while detaching the spadix from leaf base.
- In order to reduce the initial microbial inoculum, swab the excised spadix with rectified spirit in the field itself and put inside the polythene bag. Keep polythene bag with spadix inside the ice-box and bring to the laboratory.



Fig. 10. Non-destructive sampling of inflorescence from hotspot garden

### 2.2.2. Preparation of explants

- In the laboratory, the spadix (Fig. 11) is flame-sterilized after swabbing with alcohol. The base and middle portion of the rachillae (Fig. 12), which are the explants, are chopped into smaller pieces of 1-2 mm size (Fig. 13) and aseptically inoculated into culture media.





**Fig.11. Extracted spadix**



**Fig.12. Three portion of inflorescence**



**Fig.13. Inoculation of explants in medium**

### 2.2.3. Media and culture conditions

- Eeuwens Y3 medium (Annexure I) supplemented with picloram (200  $\mu\text{M}$ ), sucrose (3 %), activated charcoal (0.1 %) and agar (0.6%).
- Initially, incubate the cultures in dark with the temperature maintained at  $27^\circ\text{C} \pm 2^\circ\text{C}$  and relative humidity at 80 %.
- Sub-culture once every 30-35 days to lower levels of picloram (from 200 $\mu\text{M}$  - 100 $\mu\text{M}$  - 50 $\mu\text{M}$  - 25 $\mu\text{M}$  - 10 $\mu\text{M}$  - 5 $\mu\text{M}$  to hormone free medium).
- After 16 weeks, calli is initiated from the explants (Fig.14). Transfer cultures to illumination room ( $40 \mu\text{E}^{-2} \text{S}^{-1}$ ) with photoperiod of 16 hours
- Somatic embryo formation is achieved in regeneration medium (half strength MS medium supplemented with 1 mg/l BA) (Fig.15).



**Fig.14. Embryogenic calli from inflorescence explant**



**Fig.15. Germination of somatic embryos**

- Somatic embryo germination occurs in Y3 medium supplemented with 5 mg/l BA. Subsequent plantlet development is achieved in ½ strength MS medium supplemented with 10 mg/l BA and 5 mg/l IBA and 0.5 mg/l NAA (Fig .16).



**Fig.16. Various stages of somatic embryo formation**

#### 2.2.4. Hardening and field establishment

- Treat plantlets with minimum three opened leaves and fairly good root system with 1% Carbendazim before transferring to pots (Fig.17).
- Potting mixture consists of sterilized sand and soil in the ratio of 5:1.
- Cover plantlets initially with polythene bags and subsequently regulate relative humidity by providing the perforated polythene bags for four weeks (Fig.18).
- Transfer established plantlets to individual polybags for further hardening for 12-18

months in net house before transplanting to field (Fig. 19 and 20).

#### 2.2.5. Practical utility

- Mass multiplication of elite YLD field resistant palms.
- Mass multiplication of dwarf hybrids viz., Hirehalli X Mohit Nagar; Hirehalli X Mangala; Hirehalli X Sumangala
- Somatic embryos for *in vitro* conservation and cryopreservation

#### 2.2.6. Publications

Anitha Karun, Siril, E. A., Radha, E. and Parthasarathy, V. A.(2004) Somatic embryogenesis and plantlet regeneration from leaf and inflorescence explants of arecanut (*Areca catechu* L.). *Current Science* 86(12):1623-1628.

Anitha Karun, Radha, E., Manimekalai, R., Sajini, K. K., Ananda, K. S. and Kumaran, P.M. (2005). *In vitro* multiplication of yellow leaf disease resistant elite arecanut palm (*Areca catechu* L.). Paper (oral) presented at National Symposium on Plant Biotechnology: New Frontiers at CIMAP 18th-20th November, 2005. Abstract pp31.

Radha E., Anitha Karun, Ananda, K. S. and Kumaran, P. M. (2006) Direct and indirect somatic embryogenesis in YLD resistant elite arecanut palms. *Journal of Plantation Crops*. 34(3): 540-545.





Fig. 17. Plantlet ready for pot planting



Fig. 18. *Ex vitro* establishment



Fig. 19. Well established plantlets in polybags



Fig. 20. Field established plantlets

The arecanut farmers are facing a serious problem of crop loss due to the Yellow Leaf Disease. However there exists scope for evolving field resistant lines by making use of palms that are not affected by the disease in hot spot areas. The problems associated with controlled pollination in arecanut, together with prolonged evaluation period, delays the results from conventional breeding programmes. On the other hand, the tissue culture protocol offers possibilities of clonally propagating the field resistant palms. It is now possible to generate sufficient planting material with in a short period of time by means of arecanut inflorescence tissue culture protocol. It can also be applied for mass multiplication of hybrids of dwarf X tall varieties. Another benefit from this protocol is the possibility of cryopreservation of somatic embryos. By standardizing the tissue culture protocol, it is now possible to initiate genetic transformation studies and developing of transgenic arecanut plants.

## 2.3. Oil Palm (*Elaeis guineensis* Jacq.) tissue culture (explant: tender leaf tissues of seedlings)

### 2.3.1. Preparation of explants

- Sample the seedlings destructively by removing the outermost leaves and retaining a few interior leaves with the middle column, which contains the meristem.
- Keep the explants in a beaker to which a few drops of Tween-20 has been added and place under running tap water for half an hour to one hour.
- Keep the material in a beaker containing Bavistin (1 %) for 1 hour.
- Surface-sterilize the explants by wiping with alcohol and flaming inside the laminar air flow chamber.
- Dissect out explants of leaf lamina and leaf base (0.3 to 0.5 cm pieces).
- Inoculate leaf explants into callus induction medium.

### 2.3.2. Media and culture conditions

#### 2.3.2.1. Callus induction medium

- $\frac{1}{2}$  MS (Annexure II) + 2, 4-D (25 mg /l) + 2-iP (3 mg/l) + adenine sulphate (40 mg/l) + sucrose (3%) + phytigel (0.2 %) + charcoal (0.25%).
- Incubate the cultures in the callus induction medium (temperature  $27 \pm 2^\circ$  C; Relative humidity 80-85%) in dark.



Fig. 21. Callus induction from leaf explants after 100-120 days

- Sub-culture or transfer to fresh medium once every 30-40 days.
- Callus induction takes place after 100-120 days in *dura* and 150-180 days in *tenera* (Fig. 21).



Fig. 22. Somatic embryo formation



Fig. 23. Germination of somatic embryos





Fig. 24. Embryogenic calli



Fig. 25. Meristemoid development



Fig. 26. Shoot development



Fig. 27. Detached meristemoids in rhizogenesis medium



Fig. 28. Plantlets in rhizogenesis medium in bigger tubes

#### 2.3.2.2. Regeneration medium:

- $\frac{1}{2}$  MS + 2, 4-D (0.1mg/l) + 2-iP (3 mg/l) + zeatin riboside (1mg/l) + sucrose (3%) + phytigel (0.2%) + charcoal (0.15%).
  - Transfer callus to regeneration medium to induce somatic embryogenesis/organogenesis (Fig. 22, 23, 24, 25 and 26).
  - Maintain the cultures under illuminated condition with 16 hour photoperiod ( $40 \mu\text{Em}^{-2} \text{s}^{-1}$ ) for further differentiation.
  - Sub-culture at monthly intervals.
- If plantlets develop through meristemoid formation, transfer shoots with 3-4 leaves and having a height of 10 - 12cm to rhizogenesis medium [ $\frac{1}{4}$  Y3 (Annexure I) + IBA (5 mg/l) + NAA (1 mg/l) + Sucrose (2%) + Charcoal (0.1%)] (Fig. 27 and 28).

#### 2.3.3. Plantlet acclimatization

- Transfer plantlets with well developed shoot and roots to pots after treatment with



**Fig. 29. Well established plantlets in pots**

Carbendazim (1%) and IBA solution (1000 ppm) for an hour.

- The potting mixture consists of sterilized soil, sand and coir dust in equal proportions.
- Provide high humidity to the plantlets initially by covering them with polythene bag, then reduce the humidity gradually by making perforations in the bag and later remove the bags at night. After four weeks, remove the bags completely (Fig. 29).

- Provide MS macro nutrients ( $\text{NH}_4\text{NO}_3$ -1650 mg/l;  $\text{KNO}_3$ - 1900 mg/l;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  370 mg/l;  $\text{KH}_2\text{PO}_4$ ;170 mg/l) once every seven days.
- Transfer hardened plants to polybags consisting of potting mixture (soil, sand, coir dust and vermicompost in equal proportions).

#### 2.3.4. Practical utility

The protocol can be used for mass multiplication of elite genotypes of oil palm through clonal propagation, creating somaclonal variation for crop improvement and for *in vitro* conservation and cryopreservation.

#### 2.3.5. Publications

Anitha Karun and Sajini, K.K. (1996).  
Plantlet regeneration from leaf explants of oil palm. *Current Science* 71: 922-926

**Production of large number of oil palm *Tenera* hybrids developed (by crossing between *Pisifera* and *Dura*) is required to achieve the targeted area expansion programmes in the country. One of the constraints in area expansion is the non-availability of sufficient number of *tenera* hybrids as only the proven crosses could be used for production of planting material. By employing the tissue culture techniques it is possible to generate sufficient number of planting materials of proven *Tenera* hybrids. It also offers scope for transgenic studies in oil palm.**



### 3. EMBRYO CULTURE

#### 3.1. Coconut zygotic embryo culture

##### 3.1.1. Extraction of embryo from the nut

- The embryos were extracted with the help of a cork borer from the large soft eye of the split-opened nut (Fig. 30).
- Surface sterilize the extracted embryos (Fig. 31) with 20% sodium hypochlorite for 20 minutes. For 8 months old nuts, embryos being small in size (1.5 to 4 mm), surface sterilize only for 10 minutes.



Fig.30. Scooping embryo with the help of cork borer



Fig. 31. Scooped out embryo

- Wash the embryos thoroughly in sterile water 4-5 times and inoculate individually into culture medium.

##### 3.1.2. Media and culture conditions

- Y3 medium (Annexure I ) + sucrose (6%) + NAA (0.5 mg/l) + BAP (0.5 mg/l) + 2 g/1 charcoal (0.20%) for embryos of dwarf accessions.
- Y3 + sucrose (3%) + NAA (0.5 mg/l) + BAP (0.5mg/l) + 1 g/1 charcoal (0.1%) for embryos of tall accessions.
- Inoculate the embryos in solid retrieval medium (Y3 medium + 30g/L sucrose + NAA (0.5 mg/L) + BAP (0.5 mg/L) and incubate in the dark till germination (Fig.32) (average germination time of mature embryos is 20-25 days for dwarfs and 35- 40 days for tall)

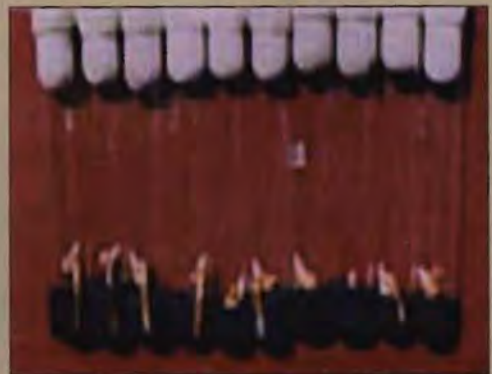


Fig.32. Germination of embryos

- Transfer to light with 16 hours photoperiod (temperature 27-29°C, Relative Humidity 70-80%)

- Subculture once every 4-5 weeks. Reduce the sucrose concentration to 2%.

### 3.1.3. Plantlet Regeneration

- Transfer germinated embryos (with two leaves and primary root, almost four months after inoculation) to liquid rooting medium (Y3 + sucrose (3%) + NAA (1 ppm) + IBA (5 ppm).
- Subculture on same medium every 4-5 weeks. Transfer to wide-mouth and longer tubes whenever necessary.
- Transfer plantlets with well-developed secondary and tertiary root and shoot system (3-4 leaves, 20-25 cm height, 5-6 ml root volume) (Fig. 33) to small pots.



**Fig.33. Plantlet in rhizogenesis medium**

- Potting mixture consists of autoclave-sterilized soil: sand: decomposed coir dust in equal proportion.

- Pre-treat the plantlets with Carbendazim (1 g /L) and IBA (1000 ppm) for 1 hour each and transfer to the pots.

### 3.1.4. Acclimatization

- Cover the plantlets (Fig. 34) with polythene bags for 2-3 weeks and keep them indoors at room temperature with artificial light



**Fig.34. Hardening of the plantlets inside the lab**

- After two weeks, harden the plantlets by gradually perforating the polythene bags to reduce humidity.
- After two weeks, remove the polythene bags completely and keep plantlets indoor for one week.
- Irrigate to keep potting mixture moist
- Transfer plantlets to bigger pots and keep them in net house with 50 % shade
- 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





**Fig.35. Well established plantlets in polybag**



**Fig.36. Field established plantlet**



**Fig. 37. Nut formation - embryo derived coconut palm**



**Fig. 38. Materials required for field collection of embryos**

- After 4-5 months, plantlets can be transferred to the field (Fig. 35, 36 and 37)

### **3.1.5. Practical Utility**

- This protocol could be applied for short term and long term (cryo-preservation) conservation and safe movement of coconut germplasm exchange

### **3.1.6. Coconut Embryo Culture Protocol for Germplasm Collection**

#### **3.1.6.1. Field collection kit**

Materials and tools (Fig. 38, 39) for collection of embryos (2000 nos.) in almost aseptic condition from the collection site are listed in Table 1.



**Fig.39. Folding type of hood**

#### **3.1.6.2. Field collection of embryos**

- A folding type portable inoculation hood made out of plexiglass is used for inoculation of embryos in the field (Fig. 39).

**Table. 1. Materials and tools required for field collection of embryos.**

S.No.	Name of the items	Quantity
1	BOX-1 (75x60x10cm)	1
2	BOX-2 ( 100x70x60 cm)	1
3	Portable folding hood and folding chair	1
4	Pressure cooker (7 L capacity)	1 no
5	Absolute alcohol	1000 ml
6	Sodium hypochlorite Solution	1 bottle (500 ml)
7	Test tube 20 cm length and 3 cm diameter)	20 nos.
8	Steel forceps(24 cm)	4 nos.
9	Stainless steel cork borer (18 cm length and 2 cm diameter)	2 nos.
10	Small knife (17.5 cm length)	1 no
11	Big knife (40 cm length)	1 no.
12	Beakers 50 , 100 and 500 ml capacity (preferably polypropylene)	1 each
13	Self sticking labels	3000 nos.
14	Scissors	2 nos.
15	Parafilm	1 roll
16	Bandage cloth (30 x 45 cm)	500 pieces
17	Plastic tray (45 x 30 cm)	2 nos.
18	Rubber bands	500 nos.
19	Non absorbent cotton	3 bundles
20	Sterile disposable gloves	200 nos.
21	Reagent bottles (500 ml and 250 ml)	1 each
22	Measuring cylinders (100 ml)	2 nos.
23	Aluminum foil	3 rolls
24	Polythene bags (45 x 30 cm)	100 nos.
25	Coconut dehusker	1 no.
26	Scalpel	2 nos.
27	Wash bottle	1 no.
28	Conical flasks (50 ml, 100 ml, 500 ml)	5 each
29	Cello tape (5 cm wide)	1 roll
30	Marker pens (4 assorted colors)	1 set
31	Carry bag (60 x30x30 cm)	1 no.
32	Sterile vials containing either sterile water or minimal growth medium	2100 nos





Fig. 40. Surface sterilization hood

### 3.1.6.3. Extraction of embryos

- Use mature nuts (11 to 12 months after fertilization) for embryo collection. (Dwarf types - 10-11 months)
- 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.
- After locating this 'eye', scoop out the embryo along with a portion of the endosperm by means of a cork borer.
- The extracted endosperm cylinders are collected in a beaker. Once all the plugs are collected from the split opened nuts, the embryo is extracted from the endosperm plug using scalpel or small knife. Extracted embryos alone are collected in a beaker containing distilled water.

### 3.1.6.4. Surface sterilization of inoculation hood

- Immediately after the extraction of embryos, the folding hood should be kept ready for surface sterilization of embryos.
- Both the inside and outside of the hood must be wiped thoroughly with alcohol for disinfection (Fig. 40).
- Disposable hand gloves must be worn while working 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.



Fig. 41. Embryo in sterile water inside the screw cap vials

### 3.1.6.5. Surface sterilization of embryos

- The surface sterilization of embryos in the field is done by 50% chlorine water (or 20% sodium hypochlorite) for 20 minutes and washing 4 to 5 times in sterile water till the traces of chlorine is removed.
- In case of immature embryos duration of surface sterilization should be reduced to 5 to 10 minutes. These embryos are very sensitive and long duration of surface sterilization leads to death of embryos.

### 3.1.6.6. Inoculation of embryos

- After proper washing, inoculate each embryo directly into a small screw cap vial (5 ml capacity) containing 1.5 to 2 ml of sterile water (Fig. 41). The sterilization of embryos and inoculation are carried out inside inoculation hood.
- An alternative to sterile water for *in vitro* active storage of embryo is half-strength Y3 medium (without sucrose) supplemented with charcoal. In either 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 (airtight) with parafilm, group the vials according to the accession and label them properly (Fig.41)
- Regeneration is as per the CPCRI embryo culture protocol. The protocol was





Fig.42. Germinating embryo

successfully applied for collecting 45 exotic accessions through 8 expeditions (Fig. 42-46)

### 3.1.6.7. Practical Utility

This protocol could be used to bring the embryos from field condition to lab condition in an aseptic manner.

### 3.1.8. Publications

- Anitha Karun, Shivasankar. S, Sajini K. K. and Saji K. V. (1993) Field collection and *in vitro* germination of coconut embryos. *Journal of Plantation Crops*, 21: 291-294.
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improvement in coconut. In coconut for rural welfare (Eds. P.K. Thampan and K.I. Vasu) published by APCC, Jakarta pp. 295-304.



Fig.43. Germinating embryos of Guelle Rose accession collected from Mauritius



Fig. 44. Plantlet with root and Shoot system



Fig. 45. Plantlet ready to transfer to poly bag



Fig. 46. Well established plantlets at ICG-SA Kidu

**It is now mandatory that exchange of coconut germplasm should only be in the form of embryo cultures for phytosanitary reasons as per Bioversity International guidelines. Increase in germination rates and reduction in transportation cost, make the embryo culture highly cost-effective. The protocol could also be used for basic studies in genetics, physiology and nutrition studies. Further it offers scope for cryopreservation of coconut zygotic embryos, a cost effective method of preserving the variability compared to the field gene bank.**

### 3.2. Arecanut (*Areca catechu* L.) embryo culture

#### 3.2.1. Extraction of embryo from the nut

- Harvest bunches seven months after pollination.
- Split open the nuts and excise the embryo (Fig. 47 and 48).



Fig. 47. Mature arecanut with zygotic embryo



Fig. 48. Extraction of embryo aseptically from the fruit

- Thoroughly wash the embryo with distilled water.
- Surface sterilize the embryos using chlorine water (50%) for 10 minutes followed by five rinses in sterile distilled water.
- Inoculate surface disinfested embryos on germination media under sterile conditions in a laminar air-flow chamber (Fig.49 and 50).



Fig. 49. Detached embryos and kernel with embryo



Fig. 50. Embryo in nutrient medium

#### 3.2.2. Media and culture conditions

- Germination medium: Eeuwens Y3 (Annexure I) + sucrose (3%) + activated charcoal (0.1%), solidified with agar (0.6%). Adjust pH to 5.8 prior to autoclaving at 121°C for 20 minutes.
- Incubate the cultures at 27±1° C in dark.
- Subculture the embryos at monthly intervals.

#### 3.2.3. Plantlet regeneration

- After 8 weeks, transfer the germinated embryos (Fig. 51) to same basal salt formulations in liquid form containing reduced (1.5%) sucrose level.
- Keep the cultures under illumination provided by white cool fluorescent tubes (Fig. 52).





Fig. 51. Germinating embryo



Fig. 53. Pot established plantlet



Fig. 52. Plantlet with shoot and root system

### 3.2.4. Acclimatization

- Transfer plantlet with balanced shoot and roots to plastic pots containing sterile soil, sand and coconut pith (1:1:1).
- Initially, cover the individual plantlets in pots with polythene bag to provide humidity for *ex vitro* establishment. Subsequently providing holes to the polybags and later remove the polythene bag completely.
- Once the plantlets have been acclimatized, transfer them to the net house for further hardening (Fig. 53).
- Hardened plantlets can then transferred to the field.

### 3.2.5. Practical Utility

- *In vitro* retrieval protocol would serve as a basis for the future *in vitro* studies of arecanut.

- It could be used in the embryo rescue of rare hybrids and *in vitro* germplasm conservation studies on arecanut and also in the areas of safe and convenient germplasm movement.

### 3.2.6. Publications

Anitha Karun, Siril, E. A., Radha, E., and Parthasarathy, V. A. (2002). *In vitro* embryo retrieval techniques for arecanut (*Areca catechu* .L). *Proceedings of 15th Plantation Crops Symposium* held at Mysore 10th -13th December, 2002. pp. 209-214.

**The arecanut embryo culture protocol could be used for embryo rescue of rare hybrids. It could also be utilized for germplasm exchange. The standardization of arecanut embryo culture offers scope for long term conservation of zygotic embryos in cryo gene banks, which will be cost effective in terms of preservation of large amount of genetic variability compared with field gene banks.**

### 3.3. Oil Palm (*Elaeis guineensis* Jacq.) *pisifera*\* Embryo Rescue Protocol

#### 3.3.1. Extraction of Embryos

- Harvest fruits 5 months after pollination
- Excise the ovules from the fruit with the help of a small knife and collect in a beaker containing distilled water
- Surface sterilize the ovules with 0.1%  $HgCl_2$  containing two drops or Tween -20 for 10 min inside a laminar air flow chamber.
- Inoculate the surface sterilized ovules on to culture medium (Fig. 54).



Fig. 54. Rescued embryo with portion of ovule

#### 3.3.2. Media and Culture condition

- Medium : Y3 medium (Annexure I)+ sucrose (3%) + charcoal (0.1%) + NAA (0.5 mg/l) and BAP (0.05 mg/l) solidified with agar (0.55%).
- Incubate the cultures in dark at  $27 \pm 2^\circ C$  with 80-85 % relative humidity.

- Transfer germinated embryos (Fig. 55) to an illuminated room with a photoperiod of 16 hours.
- Sub culture the embryos to fresh nutrient media once a month.
- Transfer plantlets (Fig. 56 ) with well-developed shoot and root system to pots containing sterile sand and soil in equal proportions



Fig. 55. Germination of embryo



Fig.56. Plantlet development

\*Based on the fruit structure, oil palm is classified as *Dura* (thick shell; less mesocarp), *Pisifera* (shell less; embryo rarely formed) and the commercially cultivated *Tenera*, the DX P hybrid (thin shell; more mesocarp (60 - 95%), with high oil content.



### 3.3.3. Plantlet acclimatization

- Transfer plantlets with balanced roots and shoot to pots after treatment with carbendazim (1%) and IBA solution (1000 ppm) for an hour.
- The potting mixture consists of sterilized soil, sand and coir dust in equal proportions
- Provide high humidity to the plantlets initially by covering them with polythene bag; then reduce the humidity gradually by making perforations in the bag and later remove the bags at night. After 4 weeks, remove the bags completely. Provide vermicompost to plantlets once in six

months. During summer months once in two days water has to be given to plants (Fig. 57-58).

### 3.3.4. Practical Utility

It could be used in the embryo rescue of rare hybrids and *in vitro* germplasm conservation studies and also useful in the areas of safe and convenient germplasm movement, etc.

### 3.3.5 Publications

Anitha Karun, Pillai, R.S.N., Radha, E. and Parthasarathy, V.A., 2001. Embryo rescue in oil palm (*Elaeis guineensis* Jacq.) v ariety *pisifera*. *International Journal of Oil Palm* 2(2): 17-18.



Fig.57. Polybag establishment



Fig.58. Field establishment of pisifera embryo derived plantlets



One of the constraints in production of *Tenera* hybrids is the non-availability of *Pisifera* palms, as germination of its embryos in natural condition is limited as it is devoid of seed coat. At present, the *Pisifera* palms are produced by selfing *Tenera* palms. Indeed it is a time consuming process and in order to identify the *Pisifera* palms, all the progenies are to be planted and observed till they produce fruits. Savings in terms of land and resources could be achieved by employing embryo rescue technique.

**ANNEXURE-I  
Y3 MEDIUM**

Sl. No.		For 1 litre (mg/l)	Stock solution for 25 litres	For making 1 litre medium
<b>MACRO - ELEMENTS</b>				
(1)	KNO <sub>3</sub>	2020	50.50 g	40 ml/L
	KCl	1492	37.30 g	
	NH <sub>4</sub> Cl	535	13.375 g	
	NaH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O	312	7.80 g	
	MgSO <sub>4</sub> .7H <sub>2</sub> O	247	6.175 g	
			<b>Dissolve in 1 litre water</b>	
(2)	CaCl <sub>2</sub> . 2H <sub>2</sub> O	294	7.35 g	20 ml/L
			<b>Dissolve in 500 ml water.</b>	
<b>MICRO - ELEMENTS</b>				
(3)	FeSO <sub>4</sub>	13.9	347.5 mg	20 ml/L
	Na <sub>2</sub> EDTA	18.5	468.5 mg	
			<b>Dissolve in 500 ml water</b>	
Dissolve each separately in 200 ml hot water. Then to Na <sub>2</sub> EDTA solution, add FeSO <sub>4</sub> solution with constant shaking.				
(4)	KI	8.3	207.5 mg	20 ml/L
	MnSO <sub>4</sub>	11.2	280 mg	
	ZnSO <sub>4</sub>	7.2	180 mg	
	H <sub>3</sub> BO <sub>3</sub>	3.1	77.5 mg	
	CoCl <sub>2</sub>	0.24	6.0 mg	
	Na <sub>2</sub> MoO <sub>4</sub>	0.24	6.0 mg	
	CuSO <sub>4</sub>	0.16	4.0 mg	
	NiCl <sub>2</sub>	0.024	0.6 mg	
<b>ORGANICS</b>				
(5)	Glycine	2.0	50 mg	20 ml/L
	Inositol	100.0	2500 mg	
	Nicotinic acid	0.5	12.5 mg	
	Pyridoxine	0.5	12.5 mg	
	Thiamine HCl	0.1	2.5 mg	
			<b>Dissolve in 500 ml water</b>	



**ANNEXURE-II  
MS MEDIUM**

Sl. No.		For 1 litre (mg/l)	Stock solution for 25 litres	For making 1 litre medium
<b>MACRO - ELEMENTS</b>				
(1)	KNO <sub>3</sub>	1900	47.50g	Dissolve in 1 litre water 40 ml/L
	NH <sub>4</sub> NO <sub>3</sub>	1650	41.25g	
	KH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O	170	4.25g	
	MgSO <sub>4</sub> .7H <sub>2</sub> O	370	9.25g	
(2)	CaCl <sub>2</sub> . 2H <sub>2</sub> O	440	11g	Dissolve in 500 ml water. 20 ml/L
<b>MICRO - ELEMENTS</b>				
(3)	FeSO <sub>4</sub>	27.8	695mg	Dissolve in 500 ml water 20 ml/L
	Na <sub>2</sub> EDTA	37.3	932.5mg	
Dissolve each separately in 200 ml hot water. Then add FeSO <sub>4</sub> solution to Na <sub>2</sub> EDTA solution with constant shaking.				
(4)	KI	0.83	20.75mg	Dissolve in 500 ml water 20 ml/L
	MnSO <sub>4</sub>	22.3	557.5mg	
	ZnSO <sub>4</sub>	8.6	215mg	
	H <sub>3</sub> BO <sub>3</sub>	6.2	155mg	
	CoCl <sub>2</sub>	0.24	6mg	
	Na <sub>2</sub> MoO <sub>4</sub>	0.25	6.25mg	
	CuSO <sub>4</sub>	0.025	0.625mg	
	COCl <sub>2</sub>	0.025	0.625mg	
<b>ORGANICS</b>				
(5)	Glycine	2.0	50 mg	Dissolve in 500 ml water 20 ml/L
	Inositol	100.0	2500 mg	
	Nicotinic acid	0.5	12.5 mg	
	Pyridoxine	0.5	12.5 mg	
	Thiamine HCl	0.1	2.5 mg	

