Annexes

Annex 1. Coconut embryo culture protocol at PCA

Field extraction of embryos

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

Pre-sterilization

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

Media preparation

A. Preparation of stock solution

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

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

Materials

Stock solutions of:

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

1. Weigh out 45 g/l sugar in the balance and dissolve in the solution above.

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

3. Adjust the pH to 5.8 using 0.1-5 M NaOH or 0.1-0.5 M HCl.

4. Add 2.5 g activated charcoal and stir.

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

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

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

8. Cool before use.

**Asseptic techniques**

**A. Preparation of sterile embryos for culture**

1. Wash the solid endosperm cylinders with tap water several times.

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

3. Immerse in 100% commercial bleach (Zonrox) for 20 minutes in a clean beaker. For embryos which were pre-sterilized already prior to storage and transport, immerse these in 100% bleach for 5 minutes.

4. Inside the laminar flow hood after sterilization, decant bleach and rinse with sterile distilled water at least 5 times.

**B. Culture of sterilized embryos**

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

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

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

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

5. Inoculate singly into test tube containing Y3 liquid medium.

**C. Culture conditions**

1. Incubate cultures at 28-30°C with approximately 4,000-5,000 lux at 9 hr photo-period (15 hr. dark and 9 hr. light).

2. Subculture to fresh medium at monthly interval.

3. Check periodically for contamination.

4. Embryos grow at different rates. Generally, 6-8 weeks after roots and shoots are formed. The earliest recorded time to transfer *ex vitro* is 4 months.

5. All together, the culture period could be a year or more.

**Screenhouse practices**

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

Procedure

1. Take out the seedlings from the laboratory to harden them in the screenhouse for one week.
2. After one week, pot them in sterilized river sand and contained in small polyethylene bags.
3. Take out hardened seedlings.
4. Wash out the media carefully. The liquid medium contains sugar that will attract ants if not washed completely. Dip the seedlings in 2.5 g/l fungicide solution, e.g. Daconil.
5. Plant to sterilized sand.
6. To maintain high relative humidity, cover the seedlings with plastic bags. Support the plastics with bamboo pegs so that they will not sag on the leaves of the seedlings. Keep them covered for 5-7 days.
7. After this period, gradually exposure the seedlings to screenhouse conditions by partially lifting the cover for a week.
8. On the third week, the plants can be fully exposed to screenhouse conditions.
9. Water the plants regularly and apply dilute foliar fertilizer solution weekly after it has developed new leaves.
10. After 3 months, transfer the plants to bigger polyethylene bags using non-sterilized soil.
11. After another 3-5 months, the plants can be transferred to the field. The plant should have 4-6 leaves by then.
12. Provide the seedlings with the necessary cultural practices for optimum growth response especially during the first three years.

Annex 2. Coconut embryo culture protocol at UP Los Baños

1. Collect endosperm cylinders with embryos.
2. Sterilize in 5% NaOCl for 20 minutes.
3. Excise embryos from endosperm cylinders.
4. Sterilize embryos in 1% NaOCl for 10 minutes.
5. Rinse three times with sterile distilled water.
6. Inoculate in liquid Y3 medium (see Annex 5) + 60 g/l sucrose.
7. Incubate in the dark.
8. After 4 weeks, transfer to solid Y3 medium + 60 g/l sucrose. Incubate at 9 hours photoperiod at 25-29°C.
9. After 4-6 weeks, transfer germinated embryos to liquid Y3 medium + 6% sucrose.
Decapitate root tips of embryos prior to inoculation.

10. For seedlings with slow shoot and root development, it is necessary to transfer to fresh medium after 4-6 weeks.

11. Well-developed seedlings with 3 to 4 leaves and profuse lateral roots should be brought to the screenhouse and subjected to natural light.

12. After 2 weeks, take seedlings out of culture medium.

13. Wash with water to remove culture medium.

14. Dip in fungicide (Dithane M-45 2 g/l).

15. Transplant to sterilized sand.

16. Cover with plastic bag and expose to 50% shade.

17. Gradually loosen/lift plastic bag.

18. After 3-4 weeks, transplant to compose sand (1:1) mixture.

19. Keep under 30 % shade.

20. After 3 months, expose to full sun conditions.

**Annex 3. Coconut embryo culture protocol at CPCRI, India**

I. **Field collections**

**Materials**

1. A folding portable inoculation hood
2. Sterile conical flasks; 250 ml 5 Nos.)
3. Beakers: 500 ml (2 Nos.)
4. Long forceps (1 No.)
5. Alcohol 95 % (100 ml)
6. Cotton/cheese cloth: 30 x 45 cms (100 Nos.)
7. Stainless steel cork borer (diameter: 0.5 cm; length 20 cm)
8. Knives: big and small
11. Parafilm: 1 roll
12. Sterilized disposable gloves
14. Rubber bands: 500 gm
15. Dehusker
16. Vials containing sterile medium
17. Measuring cylinder: 100 ml
18. Potassium permanganate; 500 gm
19. Chlorine kit klonal flask (1 liter); separating funnel
20. Gummed labels
21. HCl

Methods

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

II. In vitro culture

1. Inoculate the embryos in solid retrieval medium (Y3 + 60 gm/l sucrose + NAA (0.5 mg/l) + BAP (0.5 mg/l)) and incubate in dark until germination (average germination time of mature embryo is 20-25 days for dwarf and 35-40 days for tall) and transfer to light with 16 hr photoperiod (Temp. 27-29°C, RH 65-70%).

2. Subculture every 4-5 weeks. Reduce the sucrose concentration to 30 g/l.

3. Germinated embryos (with two leaves and primary root, after almost 4 months of inoculation) are transferred to liquid rooting medium (Y3 + 30 g/l sucrose + NAA (1 ppm) + IBA (5 ppm).

4. Subculture to same medium after every 4-5 weeks. Transfer to wide mouth and longer tubes, whenever necessary.

5. Plantlets with well-developed secondary and tertiary root and shoot system (3-4 leaves, 20-25 cm height, 5-6 ml root volume) are ready for transfer to small pots. Potting mixture consists of (1:1:1) sterilized (autoclaved) soil: sand: decomposed coir.

6. Pre treat the plantlets with Carbendazim (1 g/l) and IBA (1,000 ppm) for 1 hour each and transfer to the pots.

III. Acclimatization

1. Cover the plantlet with polyethylene bag for 2-3 weeks and keep it indoor at room temperature but with artificial light.

2. Supply Hoaglands solution once in 15 days.

3. Irrigate to keep potting mixture moist.

4. After 3 weeks, harden the plantlets by gradual introduction of perforations in polyethylene bags.

5. After 2 weeks, remove the polyethylene bags during night for 2 weeks.

6. After 2 weeks, remove the polyethylene bags completely and let it stay indoor for 1 week.

7. Transfer to bigger pots and keep in a nethouse with 50% shade.

8. After 3-4 months, transfer the plantlets to big polyethylene bags with soil and organic
manure and keep in a nethouse with 50% shade. (Total duration from pot to polybag is 5-6 months). Irrigate regularly and apply recommended dose of fertilizer, whenever necessary.

9. After 4-5 months, plantlets can be transferred to the field.

**Annex 4. Coconut embryo culture protocol at ORSTOM**

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

   The first sampling step in the field consists of isolating and disinfecting the solid endosperm cylinder. This operation is carried out in the open air on a table which had been carefully washed and disinfected with hypochlorite. Completely dehusked mature nuts (11 - 12 months) are split into two using a clean hammer. The solid endosperm cylinder containing the embryo is removed using a cork borer (diameter 20 mm). The cork borer and forceps used for this operation are previously disinfected by immersion in a bowl containing a 3% chlorogenic sodium hypo-chlorite solution. A portable gas burner is used to sterilize the instruments. Batches of 30 cylinders are immersed for 20 minutes in a 500 ml of a calcium hypochlorite solution (70% active chlorine: 45 g/l).

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

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

3. **Embryo excision before inoculation**

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

**II. Embryo culture conditions**

The culture medium used for embryo germination (MI 502) contains Murashige and Skoog mineral element (1962), Morel and Wetmore (1951), sodium ascorbate (100 mg/l), sucrose (60 g/l), neutralized activated charcoal 92 g/l (Sigma). The pH is adjusted to 5.5 before adding the charcoal and autoclaving 20 minutes at 110°C. The embryos are cultured in 24 x 160 mm test tubes containing 20 ml of medium sealed with plastic parafilm. They are incubated in a dark room at 27°C.

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

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

**III. Acclimatization procedure**

The protocol is performed in a tropical greenhouse where humidity and temperature are
controlled. Once removed from the culture medium, the plantlets are carefully rinsed with distilled water and then plunged for 5 minutes in a fungicide based on carbendazin (Benlate, 2 g/l) to prevent fungi development. They are then placed on sterile river sand. By using plastic bags (Acrylic polypropylene) for covering each plantlet during the first two weeks, it is possible to maintain maximum relative humidity conditions. Progressively, the plastic is opened. Plantlets are watered with water alone for the first month and then a nutritive solution is applied every two days.

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

- KNO3: 274.00
- Ca(NO3)2.2H2O: 1095.00
- KH2PO4: 137.00
- MgSO4.7H2O: 274.00
- (NH4)2SO4: 137.00
- KCl: 2.74
- H3PO3: 3.00
- MnSO4.H2O: 15.00
- ZnSO4.7H2O: 2.74
- (NH4)6Mo7O24.4H2O: 2.74
- H2SO4: 0.137
- CuSO4.5H2O: 1.37
- FeSO4.7H2O: 24.90
- EDTA: 26.10

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

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

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<tr>
<th>Chemical</th>
<th>PCA Y3</th>
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* except for agar, sucrose and AC
** germination medium
*** rooting