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A Guide to the Zygotic Embryo Culture of Coconut Palms (*Cocos nucifera* L.)

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A Guide to the Zygotic Embryo Culture of Coconut Palms (*Cocos nucifera* L.)

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COCONUT embryo culture has many applications, foremost of which is in the facilitation of germplasm movement (Frison et al. 1993). Embryo culture excludes the introduction of fungal, bacterial, and insect pests with the germplasm, and embryo-derived plantlets may be screened for virus (Randles et al. 1992), viroid (Hanold and Randles 1991; Hodgson and Randles 1994) and MLO (Harrison et al. 1994; Rohde et al. 1993) contamination using molecular techniques. Other advantages include obvious weight and bulk reductions, since one fruit weighs the equivalent of 10 000 coconut embryos (Harries 1982). The technique also reduces the risk of spoilage during transit which occurs with seed-nuts.

Zygotic embryo culture also has the potential for use in *in vitro* selection work for drought tolerance (Karunaratne et al. 1991), for rescuing potentially abortive types such as Makapuno (de Guzman and del Rosario 1964; Rillo and Paloma 1992b), for use with cryopreservation techniques (Assy-Bah and Engelmann 1992) and for embryo cloning (Karunaratne and Periyapperuma 1989).

It should be noted, however, that embryo culture requires specialised equipment and technical ability. It is labour intensive and slow compared with *in situ* germination and seedling development. There are alternatives to embryo culture for germplasm importation. These include pollen collection (Whitehead 1965), seed-nut importation by air (Whitehead 1966)

and seed-nut importation by sea (Le Saint et al. 1989). The quarantine risks of these methods can be minimised by following the guidelines described by Frison et al. (1993). The choice of which importation method to use should be made on the basis of technical and monetary constraints and the risk involved with germplasm importation from the particular area (Ashburner 1994).

It is assumed that the reader of this guide is familiar with plant tissue culture techniques.

Embryo Isolation and Preparation

The procedure used for the preparation of embryos for *in vitro* culture depends upon the circumstances under which they are collected. If collection occurs at a location that is remote from where the embryos will be cultured, transportation of embryos is required. The basic sequence of embryo isolation and preparation is:

- isolation of the embryo plug,
- isolation and surface sterilisation of the embryo, and
- culturing of the embryo.

Transportation of the tissue may be done between any of these stages and the methods used will be covered under the respective topics. The need for transportation is also dictated by the equipment available. It is possible to isolate the embryo plug in the field, isolate and surface sterilise the embryo in an improvised laboratory, but the culturing of embryos should take place only in a fully-equipped laboratory (Appendix 1) although some success has been achieved with field explanting with specialised equipment (Assy Bah et al. 1987). Various other protocols have been developed for coconut embryo collection (Assy Bah et al. 1987; Sossou et al. 1987; Rillo and Paloma 1992a) and reference should be made to those techniques in conjunction with the one presented here.

An improvised laboratory consists of any enclosed room which is clean and free from draughts. The laboratory should have a work bench that has been disinfected with alcohol, bleach or disinfectant. Ideally,

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there should be a spirit lamp or gas burner for sterilising instruments. Equipment for sterilising water, e.g. a pressure cooker, would be advantageous, although repeated boiling of water would suffice.

Isolation of the embryo plug

The aim of this procedure is to remove a plug of endosperm from the coconut where the embryo is found. This process may be performed either in the field, or near an improvised laboratory.

The following equipment is required to perform the operation (Fig. 1):

- bush knife or machete
- small knife or cork borer
- plunger to use with cork borer
- sharp stake to dehusk coconuts
- plastic bags to put plugs in.

The embryo plugs can be transported in these plastic bags for up to 1 day without any treatment. If transportation for longer durations is required, the method devised by Rillo and Paloma (1992a) may have to be employed. This method requires the disinfection of the embryo plugs with full-strength domestic bleach (24% active chlorine) for 20 minutes, rinsing and storage under refrigerated conditions before re-disinfection in bleach for 5 minutes under aseptic conditions before removal of the embryo. It is possible to store the plugs for up to 7 days using this method.

The embryo of coconut fruits is embedded in the solid endosperm of the coconut beneath the operculum or 'soft eye'. Its position is also indicated by a small indentation in the solid endosperm when viewed from the interior of the coconut (Fig. 2). In some cases, particularly in the small-fruited dwarf ecotypes, the position of the embryo as viewed from the inside of the nut is difficult to establish. In such cases, the position of the operculum should be used as a guide to embryo location.

- Step 1.** Select mature fruit directly from the palm or soon after it has fallen. Fruit of indeterminate age should be avoided as the embryo cannot be used once it has started to germinate. The endosperm should be fully developed, with the husk starting to brown and it should splash when shaken.
- Step 2.** Dehusk the fruit on a sharpened stake.
- Step 3.** Break the coconut around its middle with a sharp hit from the back of a bush-knife or an iron bar (Fig. 3).
- Step 4.** Working from the inside of the coconut, remove the plug of endosperm that surrounds the embryo. The plug should have a diameter of about 20 mm. A cork-borer is ideal for this job, but a sharp, thick-bladed knife may also be used (Fig. 4).

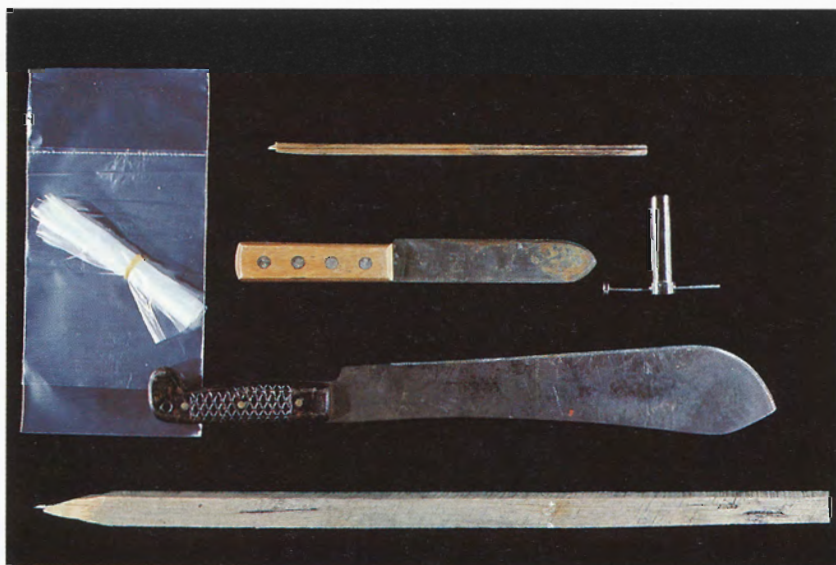


Figure 1. Equipment required for embryo plug isolation.

Step 5. Place plug in a clean container or new plastic bag.



Figure 2. Position of the embryo inside the coconut fruit.



Figure 3. Correct splitting of the coconut fruit.

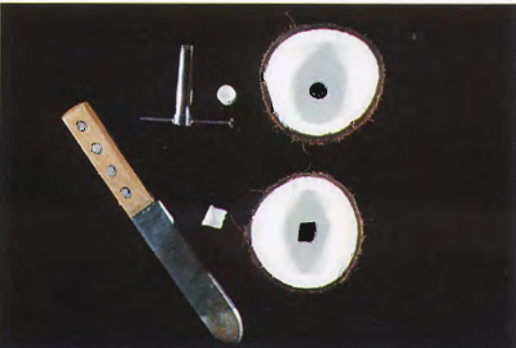


Figure 4. Removal of the embryo plug.

Isolation and surface sterilisation of the embryo

The aim of this stage is to remove the embryo from the plug of endosperm and then surface sterilise it. If this occurs at the final destination, the sterilisation must be done in a laminar flow cabinet observing sterile techniques. If further transportation is required, then this stage helps to reduce the numbers of potential contaminants that may multiply during transportation. The sterilisation procedure should then be undertaken on arrival at the ultimate destination.

Equipment/material required (Fig. 5):

- knife
- forceps
- 20 mL syringe
- bleach or sodium hypochlorite (NaOCl)
- ethanol
- culture tubes or transport tubes
- sterile water
- spirit lamp or burner.

Step 1. Wash the plugs in running water for 12 minutes (if available).

Step 2. The plugs should be rinsed in 70% (v/v) ethanol for 30 seconds (if available).

Step 3. A small cut should be made at the edge of the endosperm plug that is directed towards the embryo's position. The cut should then be opened up to expose the embryo. Other cuts may be made if unsuccessful at first. The embryo should then fall free or may be gently prised out (Fig. 6). A small, thick-bladed knife, such as a cutlery knife, is a good tool.

Step 4. Place the embryos directly into an opened syringe body directly from the plug (Fig. 7). No more than 50 embryos should be placed in each 20 mL syringe.

Step 5. Replace the plunger and draw a 1% (active chlorine) solution of sodium hypochlorite into the barrel. Eject any air and leave for 15 minutes (Fig. 8).

Step 6. Expel the chlorine from the syringe, draw in and expel two changes of sterile water. This step can be omitted if sterile water is not available (Ashburner 1994).

Step 7. Remove the embryos with forceps and place in germination media or transportation tubes (two embryos per transportation tube). The forceps should be flamed every five tubes.

Thought should be given to the method and layout used for sterilising transportation or culture tubes, to minimise contamination. Figure 9 illustrates a well devised layout. Note the following features: closeness of the flame to the working area, forceps on the right (opposite for left-handed people) to prevent continual movement over the working area, open syringe that protects embryos from falling spores. The syringe is well away from drafts created at the work area and is close to the tubes.

Transportation of embryos

Once embryos have been isolated, they can be successfully transported to another laboratory. Transportation periods of up to 6 weeks can be tolerated indicating that air-mail can be used for transportation, although air-freight is normally used to ensure rapid delivery. A modularised collection kit (Fig. 10) can be dispatched to the collection site and contains:

- forceps
- cork-borer
- disposable syringes
- transportation tubes and container
- disposable gloves for working with bleach.

In this case, transportation tubes are 1.5 mL cryopreservation tubes that have an O-ring to ensure a good seal and also are autoclavable to ensure sterility. Each tube contains 1 mL of sterile distilled water and two embryos should be placed in each tube (Fig. 11) sealed and placed inside the tube container. This

system isolates embryos from one another and effectively stops the spread of contaminating micro-organisms during transport.

Resterilisation of embryos after transportation is done as follows. Tip entire contents of transportation tube into the barrel of a syringe, the water should dribble through the bottom (Fig. 12). Continue until 50 embryos are contained then repeat steps 5–7 of the isolation procedure.

Storage of embryos

Some techniques have been developed that enable storage of embryos in a viable state for culturing at some future time. For short-term storage, the method of Assy-Bah and Engelmann (1993) can be followed. This method consists of placing the embryos on germination medium that has been gelled with 0.8% agar, but without sucrose. After 6 months of storage, 100% of the embryos can develop into plants when placed in germination media. Long-term storage can be achieved by cryopreservation of partially dehydrated mature embryos that have been placed in a medium consisting of 60% glucose and 10–15% glycerol for 11–20 hours (Assy-Bah and Engelmann 1992).

In vitro culture of embryos

The *in vitro* culture of coconut embryos requires a functional tissue culture laboratory that has facilities for the preparation and sterilisation of media, a laminar flow hood for transferring plants to fresh



Figure 5. Equipment required for isolation and surface sterilisation of the embryo.

media, and facilities for growth of the cultures. See Appendix 1 for a list of equipment required.

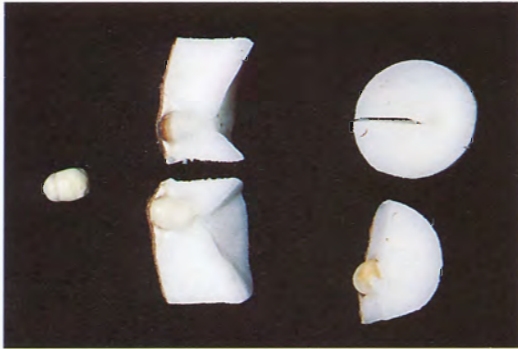


Figure 6. Removal of the embryo.



Figure 7. Embryos should be placed directly into an opened syringe body.



Figure 8. Sterilise embryos inside a syringe with 1% NaOCl.

Germination

Embryos should be placed in 10 mL of germination medium, one embryo per culture container (Fig. 13). The germination medium (Table 1), which was first used by Assy Bah (1986) consists of the macro- and micro-elements of Murashige and Skoog (1962) with the organics of Morel and Wetmore (1951). It should be noted that the medium is liquid, as coconut embryos that are germinated in a liquid medium grow more rapidly than those germinated in solid media (Ashburner et al. 1991). The embryos should be transferred to growth media immediately after they have germinated. Germination is indicated by the emergence of the plumule from within the cotyledonary sheath. This occurs after swelling of the embryo, between 2 and 8 weeks after the initial culturing. In our experience, embryos that have not germinated after 8 weeks should be discarded, as would be done with seed-nuts in a nursery.

The sucrose level of the medium is 6% (w/v), which mostly acts as an osmoticum (del Rosario and de Guzman 1976), but is also the component that stimulates germination (Ashburner 1994). Activated charcoal is essential for optimum germination in the germination medium (Rillo and Paloma 1990). No exogenous growth regulators are required during this stage. The haustorium will not develop on the embryo because of damage during surface sterilisation, but it may do so if other sterilisation protocols are followed. Germination occurs most rapidly at 30°C but will also occur at temperatures down to 25°C, though at a slower rate. No external lighting is required at this stage as weight gain is higher in embryos cultured in darkness (Rillo and Paloma 1990).

In vitro growth

After the embryos have germinated, they should immediately be transferred to growth medium (Table 2). The growth medium consists of BM3 medium (Eeuwens 1978). Germination medium may be used as an alternative growth medium with the addition of 8 g/L agar and 4% sucrose instead of 6%, but BM3 generally gives better growth in the post-germination stage (Maheswaran and Thompson 1988).

Plantlets that develop from embryos *in vitro* follow the same developmental pattern as those that develop from seed-nuts (Fig. 14), apart from lacking a haustorium. Generally, the shoot consists of four scale leaves that develop over the first 20 weeks or so, although this period may be protracted in the case of slower plantlets. After the formation of scale leaves,

the first photosynthetic leaves (or eophylls) form. It is at this stage that the plants may start looking constricted in their containers. The root system forms soon after the emergence of the shoot, but may be retarded in some individuals, and may even fail to form altogether. The primary root will grow into the growth medium and produce secondary and possibly tertiary roots. The root may also force the plantlets out of the basal medium and require pruning. Adventitious roots may form from the base of plantlets. In general, plantlets are ready to be removed from culture after 6 to 8 months. A plantlet is ready for acclimatisation when it has a root system and a shoot with photosynthetic leaves at least 160 mm in long (Fig. 15). Plantlets should be discarded if they are slow to reach this point as it is unlikely that they will successfully acclimatise.

No systematic study has been made on the lighting and temperature requirements of coconut embryo cultures. In most published studies the temperature ranges between 27 and 30°C. Coconut plantlets will develop in very low or even no light, but may have difficulty photosynthesising after removal from culture. The light from two cool-white fluorescent tubes has been found to be adequate in our studies.

Transferring the plantlets to new growth media is essential to maintain growth, especially when the plantlet is older. The sub-culture frequency should be between 4 and 8 weeks and the amount of medium should start at 20 mL for small plantlets and increase to 40 mL for larger plantlets (after 4 months of culture).

The growth of plantlets may be manipulated by adding growth regulators and changing the sucrose concentration of the basal medium. The effects of growth regulators such as auxins, cytokinins and gibberellins have not been fully evaluated because of the confounding effects of activated charcoal that adsorb organic additives. Some auxins, such as α -naphthaleneacetic acid (NAA), stimulate the production of adventitious roots as well as promoting root elongation (Ashburner et al. 1993a). Levels of NAA from 100–800 μ M may be used either continuously or for a 4 week period, the response increasing with concentration. The sucrose levels of the medium affects the shoot:root ratio of the developing plant; 4% favours shoot production whereas 8% favours root production. There is also an interaction with the effectiveness of NAA decreasing when the sucrose level of the basal medium increases (Ashburner et al. 1993b).



Figure 9. A well devised layout for embryo isolation and surface sterilisation.

Table 1. Germination medium (Assy Bah 1986).

Component	Amount	
	μM	mg/L
<i>Macronutrients</i>		
NH_4NO_3	20.6	1650
KNO_3	18.8	1900
KH_2PO_4	1.25	170
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1.50	370
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	3.0	440
<i>Micronutrients</i>		
H_3BO_3	100	6.2
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	100	22.3
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	30	8.6
KI	5	0.83
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	1	0.25
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.1	0.025
$\text{CuCl}_2 \cdot 6\text{H}_2\text{O}$	0.1	0.025
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	100	27.8
$\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$	100	37.3
<i>Organics</i>		
myo-Inositol	555	100
Na-ascorbate	463	100
Thiamine-HCl	2.96	1
Pyridoxin-HCl	4.86	1
Ca-pantothenate	2.10	1
Nicotinic acid	8.12	1
Biotin	0.041	0.01
Sucrose	-	60000
Activated Charcoal	-	2000

Table 2. Growth medium (Eeuwens 1978).

Component	Amount	
	μM	mg/L
<i>Macronutrients</i>		
KNO_3	20	2020
KCl	20	1492
NH_4Cl	10	535
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	2	312
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1	247
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	2	294
<i>Micronutrients</i>		
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	50	11.2
KI	50	8.3
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	25	7.2
H_3BO_3	50	3.1
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	1	0.24
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	1	0.24
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	1	0.25
$\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$	0.1	0.024
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	100	27.8
$\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$	100	37.3
<i>Organics</i>		
myo-Inositol	566	102
Thiamine-HCl	3	1
Nicotinic acid	8	1
Pyridoxin-HCl	5	1
L-Glutamine	685	100
L-Arginine	574	121
L-Asparagine	667	88
Sucrose	-	40000
Activated charcoal	-	2000
Agar	-	8000

Plantlets may develop abnormally for various reasons. Some abnormal growth patterns cannot be remedied and the plantlets should be removed from culture, especially those with stunted growth (Fig. 16) and senescence (Fig. 17). Plantlets may also be unbalanced in their shoot:root growth. Commonly, some plantlets fail to produce roots or fail to elongate. This can be remedied by the application of a high concentration (400–800 μM) of NAA for a period of 4 weeks which will stimulate the production of adventitious roots without affecting shoot growth. NAA can also be applied at levels of 100–200 μM continuously but this may retard shoot development

and also retard development after acclimatisation (Assy Bah et al. 1989). Too much root growth can cause shoot stunting and even death (Fig. 18) and can be remedied by root pruning and decreasing the sucrose concentration of the medium. NAA application may be required later to re-stimulate root production. Albino plantlets sometimes develop (Fig. 19) and these plants should recover if they are immediately sub-cultured to fresh media.

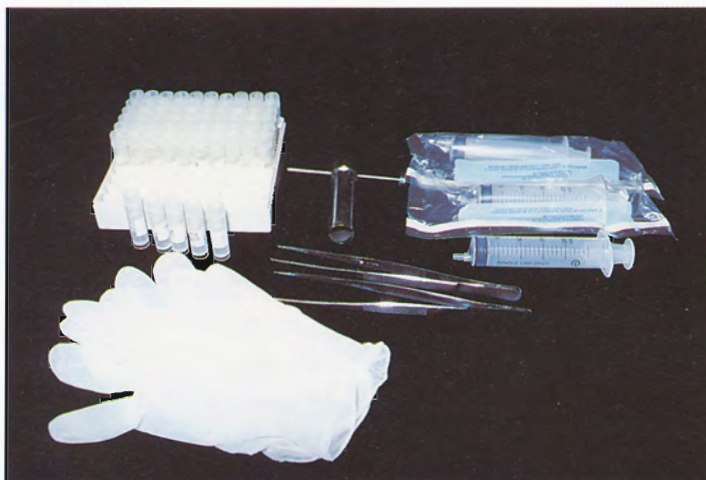


Figure 10. Modularised kit for collection of embryos.



Figure 11. Transport tubes for coconut embryos.



Figure 12. Resterilise embryos after transportation.



Figure 13. A cultured embryo before germination.



Figure 14. A normally developing embryo culture.

The equipment required to culture coconut embryos is presented in Appendix 1. It is particularly important that an isolated growth room, or cabinet, is available which can be temperature controlled. Within the growth room, a strict regime of hygiene should be maintained, particularly in a tropical environment. A negative ion generator should be installed to rid the atmosphere of dust and air-borne fungal spores. The floor of the growth room should be cleaned every day with a disinfectant solution and the walls and shelves wiped every 2 weeks. There should be limited access to the growth room and it should not open directly to the outside.

Acclimatisation

It is not possible to keep a coconut plantlet *in vitro* indefinitely, and plantlets eventually require acclimatisation. This is the process of bringing the plant out of culture and placing it in free-living conditions where it must photosynthesise and overcome challenges from micro-organisms. A plant is ready for this step when it has at least one photosynthetic leaf, an extensive root system and a shoot length of at least 160 mm. Generally, the more vigorous the plant, the easier it will acclimatise (Ashburner 1994).



Figure 15. A normally developing embryo culture, ready for acclimatisation.



Figure 16. Embryo culture exhibiting abnormally stunted growth.



Figure 17. Embryo culture exhibiting senescence. Note that it is a culture of a twin embryo.



Figure 18. Embryo culture exhibiting excessive root development at the expense of the shoot.



Figure 19. Albino embryo culture.

The most important factors to consider when acclimatising coconut plants is reducing the pathogenic load by drenching it in fungicide and maintaining humidity for the first 2 weeks because of poorly developed stomata *in vitro* (Malijan and del Rosario 1986). Shading is also required until the plants are fully acclimatised. The plants must quickly develop new leaves because those that develop *in vitro* do not photosynthesise to any great extent (Ashburner 1994).

Equipment/materials required (Fig. 20):

- clear plastic bags
- potting media (coarse sand and vermiculite/perlite or peat or coir dust 1:1)
- pots/polybags
- rubber bands
- benzimidazole (Benlate) fungicide
- shade cloth.

- Step 1.** Loosen the lid of the culture flask 3 days before acclimatisation.
- Step 2.** Remove plant from tube, wash media off roots, trim back any dead leaves.
- Step 3.** Drench in a 0.025% (w/v) solution of benzimidazole (Benlate) while wearing suitable protective clothing and gloves.
- Step 4.** Place plants in a pot/polybag with potting medium.
- Step 5.** Water, then enclose in clear plastic bag, and seal with rubber band (Fig. 21).
- Step 6.** Water three times a week. After 2 weeks, place a 20 mm hole in the top of the plastic bag to reduce the relative humidity.
- Step 7.** After another week, place a similar hole in the bag.

After a further week, cut off the top of the bag. The bag should be removed 1 week later.

Fungus growth should be controlled with more Benlate. Any dead or dying plants should be immediately removed. After 2 more months, the plants should be re-potted into larger pots/polybags and filled with sterilised sand and organic matter without disturbing the roots. Fertilising of the plants should occur weekly from the removal of the covering bag. The following fertiliser mixture has proved adequate: 0.1% NH_4NO_3 , 0.025% KSO_4 , 0.025% KCl , 0.01% NH_4PO_4 and 0.02% sequestrene. The complete acclimatisation process should be carried out under 50% shade-cloth to prevent heat and sun damage to the delicate plants. An example of a plant derived from embryo culture is shown in Figure 22 and an acclimatisation location is shown in Figure 23.

Limitations of the technique

It has been estimated that between 400 and 500 embryos must be collected in order to generate 100 plants in the field after embryo culture (Ashburner et al. 1994). This figure is based on the effectiveness of the collection technique, which varies between 47 and 92% depending on the experience of the collector (Ashburner et al. 1993b) and on the losses that occur *in vitro* due to contamination, poor growth and death, as well as the 50% loss that may occur during the acclimatisation process. However, with practice, up to 90% success rates can be achieved (Rillo and Paloma 1992b). These levels also may change drastically with the genotype of the embryo, as genotype affects *in vitro* growth and acclimatisation performance (Assy Bah 1986; Blake 1993; Ashburner 1994) with hybrids seeming to perform the best. Complete losses may also occur during the acclimatisation process if strict hygiene is not maintained. The cost of the technique, which includes the cost of the equipment, the cost of labour and training, and the cost of consumables, makes it too expensive for many small countries to use as a germplasm importation device, and therefore the technique is probably best suited to a central laboratory which handles all regional germplasm movement. This is especially true considering that germplasm importation is usually a one-off exercise. However, collection and acclimatisation procedures can be carried out anywhere.



Figure 20. Equipment required for embryo culture.



Figure 21. Coconut plantlet during acclimatisation.



Figure 22. A fully acclimatised plant derived from embryo culture.

Coconut embryo culture for germplasm collection should be used only when minimising the risk of importing diseases is most important. Importation of seed-nuts is a more reliable and economic alternative. However, embryo culture could be used when the germplasm is in a very remote location, and where transport of seed-nuts would be difficult. On a more

cautionary note, the collection of germplasm using embryo culture may cause a genetic shift in the sample population since only those individuals that have the capacity to grow *in vitro* will be ultimately used, while others, with other possibly interesting traits, will be discarded.



Figure 23. Tending the acclimatising plantlets.

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Appendix 1.

Materials required for coconut embryo collection, culture and acclimatisation

A. Embryo collection and transport

1. Bush knife or machete
2. Cork borer^{1,2}
3. Small knife
4. De-husking stake
5. Plastic bags
6. 20 mL disposable syringes²
7. Forceps²
8. Bleach or sodium hypochlorite
9. Ethanol¹
10. Culture or transport tubes²
11. Sterile water
12. Spirit/gas burner

B. Embryo culture

1. Refrigerator/freezer
2. Balance
3. pH paper/pH meter and solutions
4. Pressure cooker/autoclave
5. Culture flasks
6. Media dispenser¹
7. Laminar flow cabinet
8. Forceps
9. Scalpels and blades
10. Spirit lamp/gas burner/electric steriliser

11. Shelves with fluorescent lights
12. Thermostatically controlled air-conditioner
13. Negative-ion generator and filters
14. Glass/plastic ware
15. Pipettes
16. Magnetic stirrer/hotplate and stirring bars
17. Disposable syringes
18. Microwave oven¹
19. Media component chemicals
20. MS plant salt mixture
21. Activated charcoal
22. Sugar
23. Agar
24. Bleach/sodium hypochlorite
25. Still/Deioniser/Distilled water
26. Ethanol/Methylated spirits

C. Acclimatisation

1. Clear plastic bags
2. Potting media (sterile sand and sterile sand with organic matter)
3. Pots/polybags
4. Rubber bands
5. Benimidazol (Benlate) fungicide
6. Shade cloth
7. Soil steriliser
8. Spray gun

¹ optional

² supplied with collecting kit