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Development of an embryo culture manual and an embryo transplantation technique for coconut germplasm movement and seedling production of elite coconut types

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prepared by
Dr Stephen W. Adkins
School of Land, Crop and Food Sciences, University of Queensland

co-authors/ contributors/ collaborators
Mrs Erlinda Rillo
Mr Osmundo Orense
Albay Research Centre, Philippines Coconut Authority, The Philippines

approved by Les Baxter

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1 Acknowledgments

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2 Executive summary

The replanting of ageing coconut palms in the Asian Pacific region is now a major concern for the producing countries. This replanting will require the collecting and sharing of already existing germplasm between regions or the breeding of new high yielding and locally adapted cultivars. This collection and sharing of coconut germplasm relies on the use of an embryo culture technique which avoids the need to move the bulky fruit and also prevents the transfer of fruit-borne pests and diseases. However, the present embryo culture protocol is inefficient and consequently a new more robust embryo culture protocol is required. This new protocol would also be expected to aid the production of seedlings from the recently identified high value aromatic types and the more well known Kopyor and Makapuno coconut types.

Through ACIAR support, a new embryo culture technique has now been developed for coconut (project HORT/1998/061 - formerly CS/1998/061). The present project aims to produce a manual manuscript about this new technique. A second objective of the present project is to further develop an embryo transplantation technique which may be another way of rapidly producing seedlings of the high value coconut types.

The information generated from the previous ACIAR project (HORT/1998/061 - formerly CS/1998/061) was collected and collated to produce the new embryo culture manual manuscript. This manual manuscript then underwent a series of editing steps to accommodate the views from the partners involved in the original project. The final edited version of the manual manuscript is now available to be published in three different languages (English, Indonesian and Vietnamese) by ACIAR. The manual will find use in many laboratories including those of the Coconut Genetic Network (COGENT) International Coconut Gene banks which are located in the five main coconut-producing regions of the world.

The second part of the present project concerned the improvement of an embryo transplantation technique for the rapid production of seedlings of the high value coconut types. This part of the project took place at the Albay Research Center (ARC) of the Philippines Coconut Authority (PCA) and also involved the provision of some laboratory equipment and staff training at the University of Queensland.

A number of experiments were undertaken at ARC in attempt to improve the previously developed embryo transplantation technique. However, as yet no improvements have resulted in the germination of any of the transplanted nuts. This may relate to the fact that only poor quality fruit were available to do this work. Fruit quality had been significantly reduced by a series of severe typhoons hitting the region during the course of this work. Work is now underway to improve the technique using higher quality fruit.

The main impacts of the project have been for the Filipino project partner (ARC). The capacity of their centre to undertake coconut research has been increased enabling the present work to continue on, with some small amount of national support, after the present project finished. There is now an opportunity for the centre to take the outcomes of the project, particularly those to do with embryo culture, to the next stage of development i.e commercialisation. A pilot study will now need to take place to scale-up the approach, particularly to cater for the high value coconut types such as the aromatic types, Kopyor and Makapuno. At the same time the capacity of the partner country would be significantly improved if a genetic pool of these types of coconut could be established there. The centre could then exhibit to private investors and coconut farmers the commercial potential of the technique. This pilot project would increase the likelihood of the present project (and other previously ACIAR-funded projects) having a higher impact and for that impact to be seen more quickly.

3 Introduction

Coconut (*Cocos nucifera* L.) is the most important palm of the humid tropics. More than 12 million ha of this crop are planted in 90 countries, mainly in Asian Pacific region. The Philippines with 3.2 million ha of coconuts in plantations is the second largest coconut producing country in the world (second to Indonesia, 3.8 million ha). The crop is grown by more than 50 million resource-poor, smallholder farmers world-wide. Coconut is known as 'the tree of life' because of the many items that can be produced from it and their wide use within the local community. In addition to the traditional products of copra, coconut oil and copra meat, coconut has the ability to produce a wide variety of food and environmentally friendly non-food products, which are used domestically or exported. In some Pacific countries, these coconut products are the only source of foreign exchange earnings. Coconut has also been a stabilising factor in the farming systems of marginal and environmentally fragile environments like those found in coastal areas.

Unfortunately, world coconut productivity has been declining for decades and nearly 2/3 of the existing palms are now past their prime and need to be replaced with new, high yielding, locally adapted varieties. This requires a series of high quality breeding programs and these rely upon the availability of appropriate germplasm. At the moment the collecting and movement of coconut germplasm is undertaken using an embryo culture technique as transporting the bulky fruit is impractical and phytosanitary unsafe.

The success of the present embryo culture technique for a range of coconut types, is considered to be unreliable and therefore a new protocol has been developed (ACIAR-funded project - HORT/1998/061) with input from Australia, Indonesia, the Philippines, PNG and Vietnam. This new protocol is more efficient in its ability to produce robust plantlets, which survive planting in soil to give a high percentage of plants established. This new protocol is also applicable to the production of seedlings of the elite coconut types (viz Makapuno, Kopyor and Aromatic), which have a high economic value. However, to date, this new protocol has not been made widely available to all coconut producing countries.

Another important outcome of the earlier project (ACIAR-funded project - HORT/1998/061) was the pioneering work undertaken on embryo transplantation. With such a technique it is now possible to insert into surrogate nuts isolated embryos and nurture them into healthy seedlings. The success rate of such an approach when first developed in Brisbane, however, was low. This was put down to the fact that only poor quality supermarket fruits were available and better results would come if freshly harvested fruit in the producing country were used. Once up and running this new technique could serve as an alternative plant establishment technique to that of embryo culture, which is often tedious and expensive to undertake.

A new project (HORT/2006/006) was funded by ACIAR to further develop aspects of the past work. A manual to deliver the new embryo culture protocol will be produced in three languages (English, Indonesian and Vietnamese) and published and distributed by ACIAR. In addition, this new project would undertake further studies, using freshly produced and locally grown coconuts in the Philippines to improve the embryo transplantation technique. The present report highlights the outcomes of these two new endeavours.

4 Embryo Culture Manual

The information generated during the previous project (HORT/1998/061) was collected and collated it into a manual manuscript, to be published by ACIAR as a technical report. The manuscript has gone through a series of revisions to include contributions from all of the researchers who were actively involved in the previous project. The final version of the manual manuscript (Appendix 1) is now available to be published in English, and translated and published in Indonesian and Vietnamese and distributed by ACIAR.

5 Development of embryo transplantation technique

This portion of the project had several components; 1) capacity building, 2) training and 3) experimentation to improve the embryo transplantation procedure.

The capacity building component involved the procurement of equipment to undertake the embryo transplantation procedure (Figure 1 to 3), its shipment to ARC in the Philippines and the hiring of a research assistant to help with the implementation of the research component of the project, also at ARC, the Philippines.

Fig 1. a) Bench drill with attached nut holder, b) Hand vacuum cleaner

Fig 2. Coconut embryo transplantation laboratory. a) Preparation room; b) Clean room

Fig 3. Space in the nursery for germinating transplanted embryos

The training component involved ARC senior scientist Mr Osmundo Orense undertaking a 10 day study visit (October 2-12, 2006) to the University of Queensland to learn more about the embryo transplantation technique. As mentioned earlier, this is a new approach, first developed at UQ during the previous project (HORT/1998/061), and has potential to replace the embryo culture technique in laboratories unable to undertake tissue culture.

The experimentation component (August 2006 to December 2007) was undertaken at ARC to improve the embryo transplantation technique. First, a preliminary study was conducted to compare the germination rates of husked (intact fruit, as normally used in the coconut nursery) versus dehusked fruit. It was found that both forms germinated at the same rate (20 and 16 %, respectively). These rates are very low compared to that normally attained at ARC (i.e. 75 to 85 %). These low germination rates are due to the poor quality of the fruit which were from a remnant harvest of palms after severe typhoons passed through the region in 2006 and 2007.

A second preliminary study was undertaken to establish the basic practices for embryo transplantation and germination of the embryo-transplanted nuts. The objective was to employ the new ARC equipment, with the newly acquired technique learnt at UQ. In the first attempt, a limited number of remnant nuts of Laguna Tall were used to practice the isolated-embryo transplantation technique. To do this, a full-strength commercial bleach solution containing 5.25% NaOCl, was squirted onto the cut portion of the surrogate nut using a wash bottle for between 30 to 60 minutes (Figure 4). After this treatment the bleach was rinsed off with sterile tap water (Figure 5) and the nut allowed to air-dry. The cut shell disk was then removed to expose the underlying testa (Figure 6). The testa, including a thin portion of the endosperm, was then sliced using a sharp scalpel blade to expose the embryo (Figure 7). The embryo was then removed using a slender scalpel blade which was bent near the tip to help remove the embryo (Figure 8). Using the same blade, the germpore was widened and/or dug deeper when necessary to create a hole to accommodate the foreign embryo. After inserting the foreign embryo (Figure 9), the testa slice was restored to cover the inserted embryo (Figure 10), glue (Liquid Nail) was applied before the shell disk was replaced into its original orientation (Figure 11). More glue was then applied to seal the gap between the cut shell cap and the shell (Figure 12). The glue was allowed to dry overnight before the nut was sowing into the nursery the following day. The nuts were placed upright into a wetted germination medium (soil and coconut choir dust mix) until only the glued portion remained above the medium surface (Figure 13). A fungicide and an insecticide were sprayed over the nuts and medium soon after sowing and again every week, thereafter. The seedbeds were protected from full sunlight and rain. The moisture level of the medium was maintained by regular watering.

Fig 4. Decontamination of nuts

Fig 5. Rinsing with sterile tap water

Fig 6. Removal of sawn shell cap

Fig 7. Slicing of testa to expose the embryo (insert)

Fig 8. Removal of embryo

Fig 9. Inserting foreign embryo into the germpore

Fig 10. Putting back the sliced testa

Fig 11. Applying glue on the edge of the bored shell

Fig 12. Applying glue between the shell gap

Fig 13. Sowing of embryo-transplanted seednuts

In another study, a second kind of embryo transplantation (the insertion of an intact endosperm plug containing an embryo) was tried using the same coconut cultivar. The same decontamination, sealing and germination procedures were again applied during the transplantation steps. This time however, whole endosperm cores were extracted from the surrogate and donor nuts using a cork borer (2 cm inner diameter; Figure 14). The transplanted embryo and endosperm plug were then inserted into the surrogate nut and sealed in with glue as previously described (Figure 15).

Fig 14. Extracting the embryo disk using a cork borer

Fig 15. Inserting a foreign embryo disk into bored surrogate nut

To check if the subsequent germination responses of the transplanted nuts were due to the treatments applied, undisturbed nuts and nuts following shell manipulations (cutting, decontamination, removal, restoration and sealing) were also sown into the medium to act as controls.

As yet, many months after transplantation, no germination has been observed. Likewise, no contamination or ant damage has been noted in the transplanted nuts. Most nuts are still alive.

A refinement of the embryo transplantation technique was then attempted. This refinement involved an improved surface sterilization step where the cut nut was inverted and placed into a cup of commercial bleach solution (i.e. portion of the nut with the cut section was totally immersed into the decontaminating solution). The concentration of the commercial bleach used to do this and the duration of application were both varied to find the best combination for surface sterilization. It was found that 40 % bleach (v/v) and applied for 1 hour was the most effective method for decontamination and this procedure was then used in all subsequent experiments.

The failure of the transplanted nuts to germinate was thought due to the lack of the embryo expansion during the early germination process. This may have been a result of the poor contact of the transplanted embryo with endosperm in the surrogate nut. An experiment was then undertaken to insert the in vitro pre-incubated and expanded embryos, into surrogate nuts. To do this, embryos from mature nuts were isolated and surface-sterilised using the technique used in the new embryo culture procedure (see Appendix 1). The embryos were then incubated in a modified Y3 medium (see Appendix 1) for 3 to 5 days before being inserted in to the surrogate nuts. The same incubation medium was also used as a ‘filler medium’ to fill the gap between the re-bored germpore and embryo, after reinsertion. These transplanted nuts were then sown in a nursery as described above. However, as mentioned before, no germination has yet been recorded from them. A further identical experiment was repeated but this time using solidified MS medium (Murashige and Skoog, 1962), but the transplanted nuts still didn’t germinate.

It was then thought that the addition of a combination of plant growth regulators into the incubation medium would promote germination of the inserted embryos. An experiment was undertaken using 0.0 or 0.1 ppm BAP and 0.00, 0.05 or 0.10 ppm NAA placed into the Y3 medium in which isolated embryos were incubated for 3 to 5 days. The embryos were then inserted into the surrogate nuts. Again, it was found that the transplanted nuts didn’t germinate in the nursery.

As previously mentioned, the intact (and untransplanted) nuts sown into the nursery as controls also germinated at a much lower (16 to 53 %) than the normal ARC rate (85 %) indicating the poor quality of the fruit.

6 Problems encountered and benefits gained

In the previous project (HORT/1998/061) the success of embryo transplantation was low but significant. The lack of major success was thought due to the use of poor quality supermarket fruit as both the source of embryos as well as the source of surrogate nuts. The nuts had been imported from the producing countries, through a commercial supply chain into our supermarkets, by a technique that took weeks to complete. In addition, the transplantation technique was still in a very basic form, and therefore, a number of aspects of the technique still needed to be optimised. The use of poor quality of nuts in the present project may again contribute to the failure of the transplantation technique.

The nuts available for use came from trees severely damaged by a series by typhoons (Figure 16 and 17) and represented the best materials available at that time.

Fig 16. Coconut palms damaged by Typhoon Reming

Fig 17. Coconut tissue culture facilities damaged by Typhoon Reming: a) laboratory extension building; b) greenhouse / humidity chamber; c) nursery

A number of experiments that had been planned to help develop the transplantation technique did not eventuate due to the limited availability of coconut fruits. These lost experiments included those on nut age (both as an embryo source and as surrogate hosts), storage conditions of nuts after harvesting and before use, and the compatibility of the technique across varieties.

Despite the lack of germination of the transplanted embryos so far, significant benefits were gained during the course of the project, particularly in the area of capacity building and training at ARC. Training of a staff member at UQ and the facility development that took place at ARC has enabled them to continue the work after the end of the present project (December 2007). The extension of the work will go into a time when quality fruit will be available to be used in the study.

The manual manuscript on embryo culture manual is now ready to be produced by ACIAR (Appendix 1). This manual has been produced after extensive communication among the people involved in the previous ACIAR project. It was noted that the network (ICOPRI Indonesia, Madang Research Station PNG, Albay Research Centre Philippines and OPI Vietnam) still existed and was fully functional. Although the partners, apart from the Philippines, had no direct benefit from the project, they will benefit from the production of the embryo culture manual. The manual will be useful not only to support germplasm

movement activities but also to help produce high value coconuts such as Kopyor, Makapuno and the aromatic type seedlings, in large numbers.

7 Conclusions and recommendations

Exchange of information and views among the partners involved in the previous project (HORT/1998/061) was encouraged during the present project and was useful for the construction and editing of the new embryo culture manual. The manual is now ready to be produced in three different languages (English, Indonesian and Vietnamese). The partners could be involved in the translation process and the future distribution of the manual on completion.

A new capacity, to undertake work on embryo culture and embryo transplantation, was established at ARC. This was achieved by running a training workshop at UQ, by the improvement of tissue culture facilities at ARC, and by continuous, on-the-job training for a local assistant at ARC.

At ARC a number of experiments were undertaken in an attempt to improve the embryo transplantation technique. However, to date no transplanted nuts have germinated. The nuts used in these experiments were the remnants of a series of severe typhoons which hit the region, destroying coconut trees as well as ARC facilities, during the period of this project. This poor nut quality will have contributed to the poor progress in the germination of the transplanted embryos. Several planned experiments were also a victim of the low number of available coconut fruits to study.

In the future, some basic research is needed to further establish the technique of embryo transplantation. The work should be focused on excised embryo transplantation (rather than plug transplantation) because this is the method that shows the greatest chance to succeed. In addition, the physiological responses of the excised embryo to transplantation should be studied. This could include studying water uptake, the changes that go on in the contact area between the inserted embryos and the endosperm, and food mobilization and transfer to the embryo. Compatibility of the endosperm with the foreign embryo, in terms of their genotype, age, and pre-storage treatment, should also be examined.

The two projects (the present and the previous one HORT/1998/061) have generated much knowledge and have increased the capacity of the coconut research teams in Indonesia, Philippines, PNG and Vietnam. The new embryo culture manual is now ready to act as a comprehensive reference document for these teams. The positive outcomes of the research to date on embryo culture allow the coconut research teams to move to the next stage of development - commercialisation of the technology. A pilot project is therefore recommended to be set up in the producing country(ies) to further develop the technology and to demonstrate that large scale production of high value coconut cultivars (Kopyor, Makapuno, aromatic) is possible. The establishment of a genetic pool (a germplasm collection) of these and other useful coconut types should be undertaken in the early years of such a project and this would be to help build breadth to the commercialisation. The possibility of crossing Kopyor or Makapuno with the aromatic coconuts should also be explored before the new embryo culture protocol is used to rapidly multiply up the new hybrid seedlings. The success in production of Makapuno seedlings, on a large scale through embryo culture, can already be seed in the Philippines, and this successful venture should be used to provide further insight into how up scaling of these approaches to a commercial level can take place. Some additional research activities will be required to fine-tune the protocol and therefore the involvement of the experienced Australian team is certainly required. The private sector and farmers should be encouraged to participate in the project right from its coinception. However, unless a pilot project is initiated to demonstrate commercial potential, the likelihood of the

two projects (and some other relevant previous ACIAR projects) providing impact to the farmers and other stakeholders in coconut producing countries will continue to be low. The low impacts of ACIAR-funded coconut projects have been reviewed elsewhere (see Samosir et al. 2006).

Another impact of the present project's embryo culture protocol will be on the way coconut germplasm is moved between laboratories, at both the national and the international level. Therefore the embryo culture manual should be made available to all potential users of the technique by ACIAR. These include the International Coconut Genetic Resources Network (COGENT) which is establishing an International Coconut Genebanks to conserve coconut germplasm around the world and make it available to other interested countries. It is recommended to promote the dissemination and adoption of the new embryo technology through workshops provided by the teams that developed it.

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9 Appendix

Appendix 1: Embryo culture manual

A New Embryo Culture Protocol for Coconut Germplasm Conservation and Elite-type Seedling Production

Yohannes M S Samosir
Nurhaini Mashud
Hengky Novarianto
Vu Thi My Lien
Erlinda Rillo
Pablito Magdalita
Olivia Damasco
Alfred Kembu
Mathias G Faure
Stephen W Adkins

Australian Centre for International Agricultural Research
Canberra
2008

1 Introduction

Coconut (*Cocos nucifera* L.) is regarded by many to be the most important tropical palm, however producers of this plant still experience low, and ever decreasing yields. Most producers are tending ageing palms that were planted many years ago using unselected types. These old plantations need to be replanted with the new, higher yielding and disease-resistant types. Consequently there is a need to develop new locally adapted, but improved types. This is possible if use is made of the wide range of coconut germplasm that is available from various locations from around the world. However, due to a number of factors including natural disasters, droughts and loss of growing land to the expansion of urban and other agricultural needs, this valuable coconut germplasm is being lost rapidly.

In the past decade, there has been an effort by The Coconut Genetic Resources Network - International Plant Genetic Resources Institute (COGENT-IPGRI) to finance and establish a multi-site, international coconut germplasm conservation bank (ICG). This network of sites, many of which are already operating, are in the five COGENT regions, namely; Southeast and East Asia, South Asia, the South Pacific, African and the Indian Ocean, and Latin America and the Caribbean. It is anticipated that this network of coconut genebanks will ultimately house the majority of the world's coconut germplasm and safeguard it for future use in the coconut industry.

To help set up these conservation banks for the ICG network or for other coconut germplasm collection and establishment programs, a safe and reliable mechanism for collecting, moving and re-establishing coconut germplasm is required. The collecting and movement of whole coconut fruits is clearly impractical due to their size and the fact that transferring uncleansed fruits is phytosanitary unsafe.

For these reasons the collecting of coconut germplasm, either as individual embryos or as embryos contained within a small plug of endosperm tissue, and then moving them as in vitro cultures, has become a much more practical way of transporting coconut germplasm. The embryo of a typical nut weigh up to 10, 000 times less than the intact fruit from which they are isolated (Harries 1982) and carry no known disease vectors.

The *in vitro* culturing of coconut zygotic embryos has been achieved on numerous occasions (De Guzman and Del Rosario 1974; Assy Bah 1986; Rillo and Paloma 1992a; Samosir et al. 1999). Such techniques are also useful for rescuing embryos from the high value coconut mutant types (eg. Makapuno, Kopyor), which have a jelly-like, non-functioning endosperm tissues (Rillo and Paloma 1992b), and establishing seedlings from them. The technique can also be used for *in vitro* selection for various whole-plant traits (eg. drought tolerance; Karunaratne et al., 1991) and for cryopreservation of coconut germplasm (Assy-Bah and Engelmann 1992; Sisunandar et al., 2005).

Up until now a standard, internationally recognised coconut embryo culture technique has been used to establish germplasm collections and to produce high value seedlings from the mutant coconut types. Known as the 'hybrid embryo culture technique' (Batugal 2002) this technique has been used for the production of coconut palms, at their destination after international exchange. Although high germination rates are possible in vitro using this protocol (Engelmann and Batugal 2002) large discrepancies have been noted between different laboratories and this makes the protocol inefficient and unreliable.

Many aspects of the *in vitro*-grown plantlets' physiology are not optimal and this is thought to contribute to the low rate of plantlet acclimatization and establishment ex vitro. The physiological traits of the seedlings that are most likely affected by the technique are the development of the root system, the capacity to undertake photosynthesis, and the susceptibility to infection.

The newly developed protocol takes care of these and other potential seedling development problems and has been the result of work undertaken by a team of scientists, from different countries (Australia, Indonesia, PNG, Philippines, and Vietnam), and working over a 3 year period. Some significant improvements have been made to the protocol which can be used both for coconut germplasm movement and re-establishment and the production of elite mutant type seedlings, such as Makapuno and Kopyor.

2 Objectives

The objectives of the work undertaken to produce this manual were to develop new and improved protocols for the:

- isolation and transport of coconut embryos
- initiation of *in vitro* coconut embryo cultures
- maintenance of coconut embryo cultures until the seedlings produced are ready for establishment in soil
- acclimatization of the coconut seedlings produced
- maturation of the coconut seedlings in soil in a nursery.

3 Materials, equipment and facilities

3.1 Plant materials

Using embryos of an appropriate age (*ca*. 10 to 11 months after pollination) is an important factor if the highest possible rate of seedling production is to be obtained. At this stage of development, 10 to 11 month old embryos are contained in fruit that have an exocarp that is just starting to turn brown in colour (Figure 1). The basic rule that can be applied during germplasm collecting trips is that the harvesting of embryos from a bunch can proceed if at least one fruit has attained this brown colour stage. Other coconut fruit selection practices should also be applied, particularly those to do with fruit health and only the highest quality fruit should be used for embryo isolation. However, fruit should not be rejected if they do not have ‘a water-splashing sound’ when shaken. Such a selection step is often used in the nursery when selecting fruit for germination but has no role in the field. Fruit harvested at 10 to 11 months of age may not produce such a sound upon shaking because their cavities may be completely full of water.

The shortest possible time from harvesting of fruit to the isolation of the embryo and its inoculation onto a tissue culture medium is preferable. When collecting germplasm from remote areas it is recommended to collect ca. extra 20 % more embryos than is needed. This is to compensate for the loss of viability of some of the embryos during the transport step to the laboratory. For this transport step, the embryos can be moved either embedded in an endosperm cylinder or plug (Rillo and Paloma 1992) or as isolated, naked embryos (Ashburner *et al*. 1995; Samosir *et al*. 1999).

3.2 Equipment

The basic equipment and facilities needed for the tissue culture steps include a culture room, a laminar flow cabinet, an autoclave, a dissecting instrument kit, culture vessels and various media. A cork borer (No. 10 or bigger, Figure 2) is necessary for the extraction of the endosperm plug (containing the embryo) from the nut. The cork borer can be replaced with a sharpened metal pipe that is pushed into the endosperm using a wooden hammer, if a cork borer is not available.

Polycarbonate culture vessels (2.5 cm diameter and 8 cm high, Figure 3a) are normally used for the germination step and early seedling maintenance until at least one unfolded leaf has been produced on the seedling. Bigger vessels are then needed for the subsequent steps as the seedlings grow. A double vessel system, produced by inverting a second culture vessel over the first, the vessels being joined at the point of where their rims touch, is used for the largest seedlings (Figure 3b). Alternative to this culture vessel system, is one that uses a clear polyethylene plastic bag (autoclavable) placed over the top as an extension of the culture vessel (Figure 3c).

A carbon dioxide (CO2) atmosphere enrichment system (Figure 4) is used as an important improvement over the traditional method of continuing the incubation of the seedlings under an inverted clear plastic bag. The CO2 atmosphere enrichment system can be constructed in several ways but a very efficient laboratory unit can be made from clear perplex plastic sheets (0.6 cm thick) glued together.

Following on from this laboratory CO2 atmosphere enrichment step, seedling acclimatisation takes place. To do this an acclimatisation box (Figure 5a) is used to complete the hardening-off process in a screen house prior to the seedlings being planted out into a screen house. The acclimatisation box creates a high relative humidity around the seedling to support its development while providing a sufficiently large headspace for further growth. The box is constructed from a wooden framework covered with a clear plastic sheet, having four holes cut into it (10 cm diameter, two per side). The four holes provide some exposure to natural screen house conditions and therefore promote the seedling harden-off process. A typical box would be 100 cm long, 22 cm wide and 30 cm high. The box is placed in an elevated platform inside the screen house to access sunlight. A bigger box or a clear plastic covered tent (Figure 5b) could be used when dealing with large numbers of seedlings. The plastic cover on the box or the tent can be removed from time to time for seedling management operations such as watering or pest control.

3.3 Medium and reagents

The medium formulation developed for coconut embryo culture (Table 1) is based on Y3 minerals (Eeuwens 1976) with the modification of vitamins and iron.

Table 1. Medium components and formulation for coconut embryo culture

Compound	Chemical formula	Amount (mg/L)
Macronutrients (Y3)		
Potassium nitrate	KNO_3	2020.00
Potassium chloride	KCl	1492.00
Ammonium chloride	NH_4Cl	535.00
Sodium dihydrogen *ortho*-phosphate	$NaH_2PO_4.2H_2O$	312.00
Calcium chloride	$CaCl_2.2H_2O$	294.00
Magnesium sulphate	$MgSO_4.7H_2O$	247.00
Micronutrients (Y3)		
Manganese sulphate	$MnSO_4.4H_2O$	11.20
Potassium iodide	KI	8.30
Zinc sulphate	$ZnSO_4.7H_2O$	7.20
Boric acid	H_3BO_3	3.10
Cupric sulphate	$CuSO_4.5H_2O$	0.250

Cobalt chloride	$CoCl_2.6H_2O$	0.240
Sodium molybdate	$NaMoO_4.H_2O$	0.240
Nickel chloride	$NiCl.6H_2O$	0.024
Ferrous sulphate [1)]	$Fe_2SO_4.7H_2O$	41.70
Disodium ethylene diamine tetra-acetic acid [1)]	Na_2EDTA	55.80
Vitamins and amino acid(UPLB+ARC)		
Pyridoxine HCl (Vitamin B_6)	$C_8H_{11}NO_3.HCl$	0.05
Thiamine HCl (Vitamin B_1)	$C_{12}H_{17}ClN_4OS.HCl$	0.05
Nicotinic acid (Vitamin B_3)	$C_6H_5NO_2$	0.05
Calcium -D-pantothenate (Vitamin B_5)	$(C_9H_{16}NO_5)_2Ca$	0.05
Biotin (Vitamin H)	$C_{10}H_{16}N_2O_3S$	0.05
Folic acid (Vitamin B_c, Vitamin M)	$C_{19}H_{19}N_7O_6$	0.05
Glycine	$C_2H_5NO_2$	1.00
Others		
Abscisic acid (ABA) [2)]	$C_{15}H_{20}O_4$	0.25
α-Naphhthaleneacetic acid (NAA) [3)]	$C_{12}H_{10}O_2$	18.6
Sucrose [4)]	$C_6H_{22}O_{11}$	60.0, 45.0, 25.0
Agar (only for first and second subcultures) [5)]		7.0
Activated charcoal [6)]		1.0
pH		5.6

[1)] Iron chelate stock solutions are prepared in separate stock bottles
[2)] Filter-sterilised ABA is added to the germination culture medium to enhance germination.
[3)] NAA is applied for 1 month only to promote root formation and development.
[4)] Low grade sucrose can be used. Sucrose (60 g L^{-1}) is used from culture initiation until shoots and roots have developed (the first 3 to 4 months of culture) then a lower level of sucrose (45 g L^{-1}) is used prior to a final reduction (to 25 g L^{-1}) for the last two subcultures.
[5)] Subsequent subculture steps should be undertaken using a liquid medium
[6)] Use acid washed activated charcoal. For consistent results use the same brand during the whole culture process.

3.4 Culture incubation conditions

For the best growth, cultures are incubated at 28 to 30 0C under illuminated conditions (*c*. 40 μmol m-2 s-1) with a 14 hour light, 10 hour dark photoperiod. Red light enrichment (at c. 660 nm) may be added to promote seedling growth. A higher light intensity (*c*. 90 μmol m-2 s-1) should be used when growing the seedlings under photoautotrophic conditions (see Section 4.3.3.).

4 Procedures

4.1 Preparation, reagents and media

For the general procedures of stock solution and media preparation, please consult a standard tissue culture text (eg. George 1993). Separate stock solutions (each 10 x the final concentration) of the six macro salts are recommended to avoid precipitation. Micronutrients, except iron, are most conveniently stored in stock solutions that are 100 x the final concentration needed.

To obtain the most consistent results, liquid medium (10 mL) is used in the tube (25 x 150 mm) for culture initiation, solid medium (15 mL) is then used, followed by liquid medium

(80 mL), in the last subculture in the bigger vessels (see Section 3.2) In all steps, the media should be kept stirred to distribute the activated charcoal (acid washed, plant cell culture grade) while dispensing takes place.

4.2 Preparation of plant materials

1. The dehusked nuts are split and a portion of the endosperm, containing the embryo (endosperm cylinder) is removed using a surface sterilised cork borer.
2. The endosperm cylinders are placed in a clean vessel containing fresh coconut water, or sterile water, as an incubation medium.
3. In the laboratory, the endosperm cylinders are washed under clean water and rinsed in ethanol (95 %) for 30 s.
4. The cylinders are then surface sterilised using fresh, full strength commercial bleach (normally containing 42 g L^{-1} sodium hypochlorite) for 20 minutes.
5. They are then rinsed three times in sterile water in a clean room or laminar air flow cabinet, to minimize the potential for recontamination.
6. For transporting, the sterilized cylinders are then placed into a sterile plastic bag with cotton wool moistened with sterile water. The bag is the placed inside a Styrofoam or thermally insulated box.
7. To slowdown degradation of the materials during the transport procedure, the cylinders are kept cool using a small quantity of ice placed around the bags inside the box mixed in with further cotton wool packing.
8. The ice is replaced, as necessary, during the trip back to the laboratory. Ice will not be necessary if a portable refrigerator, connected to the car power supply, is used.
9. An alternative method for transporting the germplasm is to excise the embryos from the endosperm cylinders in the field and to transfer only the embryos to the laboratory. Once isolated, the embryos are surface sterilised using fresh commercial bleach (10 % for 1 minute), washed three times with sterile water and then placed in a tube containing sterile ascorbic acid (10 mL of 1 mg L^{-1}). The containers are then placed in the Styrofoam box containing ice or into a portable refrigerator as described above.
10. Transport of the embryos is done in the shortest possible time. This should not exceed 4 days including the collecting and processing time.

4.3 Protocols

The stages of culture explained below are summarised in a flow chart provided as an A3 size poster which should be displayed in the laboratory for easy reference.

4.3.1 Stage 1 - The start up culture

1. In the case when endosperm cylinders are transported, upon arrival in the laboratory they are re-surface sterilised and rinsed in sterile water (Steps 3 and 4, Section 4.2).
2. Working in a laminar air flow cabinet, the embryos are excised from the endosperm cylinders and placed in sterile Petri dishes.
3. In the case when isolated embryos are transported, upon arrival in the laboratory they are transferred to a sterile flask and wash three times with sterile water then transferred to sterile Petri dishes.
4. Working in a laminar air flow cabinet, the embryos from both methods of transport are surface sterilised with 10 % fresh, commercial bleach for 1 minute and then rinse with sterile distilled water three times.

5. The embryos are then transferred to sterile Petri dishes lined with filter paper to absorb any free water that may still be on their surface.
6. The embryos are then individually inoculated into test tubes containing a liquid medium supplemented with ABA (0.25 mg L^{-1}). ABA is needed to get uniform germination particularly when using embryos from different fruit ages. Addition of 10 µM GA_3 to replace ABA may be necessary when working on varieties with a low germination rate.
7. The embryos in liquid medium are then incubated in the dark for 1 month.
8. After this time, the haustoria of the embryo may (when it has grown big enough to obstruct transfer) or may not be removed (decision of the operator). The remaining portion is then transferred to a solid medium (same medium as above but containing 2 % agar and with no ABA) and subcultured every month until they have shoots and roots (in few cases, roots may not develop).
9. The germinated seedlings are then placed under illuminated conditions (see Section 3.4) once they have attained at least 1 cm shoot growth.
10. All ungerminated embryos are then discarded at the end of the 12 week incubation period.

4.3.2 Stage 2 - Early seedling growth (up to the 1-leaf stage)

1. The seedlings are sub-cultured into a liquid medium of the same formulation but contain 100 µM NAA (to promote root formation and development).
2. After 1 month in the root-promoting medium, the seedlings are subcultured into a liquid culture medium without NAA.
3. They are then subcultured every month into fresh medium until they have one unfolded leaf at which stage they are then transferred into either a conventional embryo culture growth condition or a photoautotrophic environment (if an apparatus is available to do this) (Section 4.3.3).

4.3.3 Stage 3 - Later seedling growth (up to the 3-leaf stage)

Conventional embryo culture

1. To economize on the culture medium, the monthly sub culturing of the seedlings is continued onto fresh liquid medium in long test tubes.
2. When the seedlings have produced two expanded leaves and secondary roots have formed on the primary roots, they are transferred into bigger vessels (see Section 3.2) and the height is extended using autoclavable clear plastic bags so that the seedlings can have enough headspace for normal growth.
3. At the time of the final subculture, root pruning may be necessary to ensure good contact of the shoot base and roots with the culture medium is retained. If this is not done, the roots often push the shoot up off the medium causing a low degree of contact with the medium which results in poor culture growth. The locally available substrate material, such as coir dust (sterilized), can be used in the medium to promote root development.
4. Seedlings with 3 to 4 expanded leaves and roots with secondary and tertiary rootlets, are potted out. All together the culture period could be up to a year or more.

Photoautotrophic using CO_2-enriched conditions

Instead of following the conventional embryo culture approach described above, when the seedlings have produced at least one unfolded leaf, they can be transferred into a photoautotrophic system and CO_2-enrichment conditions applied (Figure 4). The medium used in this system is one with Y3 minerals with Fe-EDTA, but without sucrose.

1. The seedlings, especially roots, are washed carefully with tap water
2. The seedlings are then placed into a Benlate^{TM} fungicide solution (2 g L^{-1}) for 15 minutes.
3. They are then transferred into 100 mL pots containing 10 g of an autoclaved vermiculite, or a coconut coir dust substrate, soaked with Y3 minerals.
4. The pots are then placed into 500 mL culture vessels with 40 mL of a liquid medium containing Y3 minerals.
5. The vessels, without lids, are then placed into a CO_2-growth box.
6. The box is then fogged with CO_2 (1600 ppm) for the illuminated portion of the photoperiod and during the dark portion with ambient air.
7. The vessels are topped up with Y3 minerals as necessary (normally every week) and the Y3 mineral solution is completely replaced every month.

4.3.4 Stage 4. Seedling acclimatization and pre-nursery care

Conventional embryo culture

1. The vessels are taken out of the laboratory and placed into a screen house for 1 week to start the hardening off process.
2. After 1 week in the screen house, the seedlings are removed from the vessels and washed with tap water
3. The seedlings are then dipped in a Benlate^{TM} fungicide solution (2 g L^{-1}) for 15 minutes and then individually planted into sterilized potting mix (garden soil and coconut coir dust in a 1:1 ratio) held in plastic bags and soaked with water.
4. The plants are placed in an acclimatisation box (Figure 5) where they are kept covered for 3 to 4 weeks or until the seedlings have recovered from *in vitro* conditions
5. After this period, the seedlings are gradually exposed to the screen house conditions by partially lifting up portions of the plastic lid of the acclimatisation box.
6. The plants are then fully exposed to screen house conditions within a 1 or 2 week period.
7. The plants are watered as required and a dilute foliar fertilizer solution is added to their leaves each week.
8. The seedlings are then maintained using common coconut nursery practice conditions, including those of pest control, until they are ready for field planting.

Photoautotrophic culture using CO_2-enriched conditions

When the seedlings have reached the 3 to 4 expanded leaf stage (normally after 2 to 3 months of growth under the CO_2-enticed conditions), they are ready to be transferred to the nursery.

1. At the 3 to 4 expanded leaf stage, the CO_2 fogging is stopped and the seedlings are gradually exposed to ambient conditions, by partially opening the lid of the CO_2 box, over a 2 week period.
2. The vessels are refilled with Y3 minerals when necessary.
3. After 2 weeks the seedlings are transferred to the screen house and planted into sterilized potting mix contained in black plastic bags.
4. A slow release fertilizer is added and a dilute foliar fertilizer solution is applied weekly for the first month.
5. The soil is watered when needed and appropriate pest control applied.

4.3.5 Stage 5. Seedling transfer to the nursery

After 3 months of acclimatisation in the shade conditions the seedlings are ready to be transferred to the nursery.

1. The seedlings are planted into bigger polyethylene bags containing non-sterilized coconut coir mixed with soil.
2. The bags are then placed into a nursery and provided with shade.
3. Follow the normal practices for coconut nursery management, the plants are watered, fertilised and pest control applied.
4. After another 3 to 5 months, when the plants have 4 to 6 leaves, with at least one of them with leaflets, the seedlings are transferred to the field and cared for using normal field planting protocols.

5 Observations

The embryo of the coconut seed is embedded in a solid endosperm and is to be found under the operculum, or the ‘soft eye’, in the hard endocarp. Embryo injury during the isolation process should be avoided and if injury does occur, the injured embryos must be discarded.

Some of the embryos will germinate within the first month after the start of culture. The germinated embryos are transferred onto the solid culture medium with care to ensure they end up in the appropriate orientation, with the roots and shoots growing in the correct direction.

The roots may force the young plantlets up off the media and therefore the roots may require pruning. Root pruning, however, is not needed when growing the seedlings under photoautotrophic conditions as this condition uses vermiculite as the supporting medium. In vermiculite the root system develops well and the ‘lifting up’ phenomenon is observed.

Rigorous exclusion of all potential contaminating micro-organisms is necessary, and cross contamination, from seedling to seedling during the subculture procedure must be avoided. Cleanliness, efficient organization and routine sterilization of all materials and equipment will reduce the risk of contamination.

For various reasons embryos and plantlets may develop abnormal features. These may include hyper-hydrated tissues or stunted embryo growth. In most cases these abnormalities are unable to be reversed and therefore the embryos need to be discarded. In addition, ungerminated embryos should be removed from the protocol after 12 weeks so that medium resources are only expended upon vigorous embryos.

6 Results

Depending on the coconut genotype cultured, germination rates in vitro will range from ca. 60 to 85 %. A further loss of 10 % may be expected during the steps leading up to successful acclimatization of the seedlings. This does not include the small losses that may stem from seedling contamination which may result from poor aseptic technique in the early stages of the procedure. Further small losses may also occur during the establishment of the seedlings in soil in the shade house. The rate of success of this new system is much improved over that of the hybrid embryo culture system and is applicable to a much wider range of coconut types.

The use of an atmospheric CO_2-enrichment system has been shown to significantly reduce the rate of seedling loss during the acclimatization steps. In addition, the time the seedlings are in in vitro conditions can be significantly reduced (from 1 year using the old hybrid EC approach to about 4 months using the new protocol). When seedlings reach the one unfolded leaf stage, they are ready for photoautotrophic growth using the CO_2-enrichment system. These steps may only take 2 further months before the seedlings are ready for the acclimatisation step and transfer to the screen house.

The photoautotrophic steps, however, requires additional equipment, materials and some degree of operator skill. The cost for this extra equipment, however, may be recovered as there is a saving to be made in using less media and because more seedlings are produced at the end of the procedure.

7 Application to mutants

A significant proportion of the work undertaken at the Albay Research Station of Philippines Coconut Authority (PCA) (Figure 6) and at the University of Queensland (UQ) to develop the new protocol was on Laguna Tall which also bears the mutant Makapuno coconut. Other materials used in the study were Malayan Yellow Dwarf (MYD), Nias Yellow Dwarf, Mapanget Tall, Bali Tall, PNG Brown Dwarf and the aromatic coconut types from Vietnam. In the case of the aromatic types, the application of 2 mg/L IBA instead of NAA (see Table 1) should be used to promote seedling root growth. In this case, IBA should be applied through the whole subculture process up until the first leaf is formed. In most studies MYD was used as a standard (control) material. As yet, there has been no comprehensive evaluation of the new protocol applied to mutant coconut types such as Makapuno and Kopyor. However, from the results generated so far and the fact that the embryos of the mutants have a similar response in vitro as ordinary embryos, the new protocol is therefore considered to be applicable to all types of coconuts including the mutants. Scaling up research is recommended to make the protocol more efficient and economically viable.

8 Safety issues

The steps described in this manual must be followed in accordance with the appropriate safety standards that will be in operation in the laboratory where the work is to be undertaken. Extra precaution needs to be followed when installing and using the CO_2-enrichment system. CO_2-enrichment systems have been in use in plant nurseries for decades and more recently in tissue culture laboratories. The CO_2-enrichment system developed for use with coconut is best described as a closed system. Using such a system, CO_2 can leak from the apparatus and accumulate in the culture room. Therefore, regular checks on the tubing and connections need to be made to reduce this leakage. The maximum CO_2 concentration that is allowed to accumulate in a building under Australian laboratory standards is 5,000 ppm. Although the gas itself is not toxic, it can cause O_2 deficiency and asphyxiation. It is therefore recommended that a monitor be used to measure the CO_2 concentration in the culture room and for action to be undertaken if such a level is reached.

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10 The protocol at glance

Figure 1. The ideal stage of development of coconut fruits for zygotic embryo isolation and culture is when several fruit in the bunch have just turned brown in colour. At this stage, their age is estimated to be 10 to 11 months after pollination.

Figure 2. Cork borer used to remove the endosperm plug from the split nut.

(a) (b) (c) (d)

Figure 3. The different types of vessels used in the embryo culture of coconut. Polycarbonate culture vessels (diameter 2.5 cm and height 8 cm) are normally used for the first step until at least one unfolded leaf has been produced (a). Bigger vessels are needed for the subsequent steps as the seedlings grow (b). A double vessel system is used for the largest seedlings (c). An alternative to this culture vessel system, is one that uses a clear polyethylene plastic bag (autoclavable) placed over the culture vessel (d).

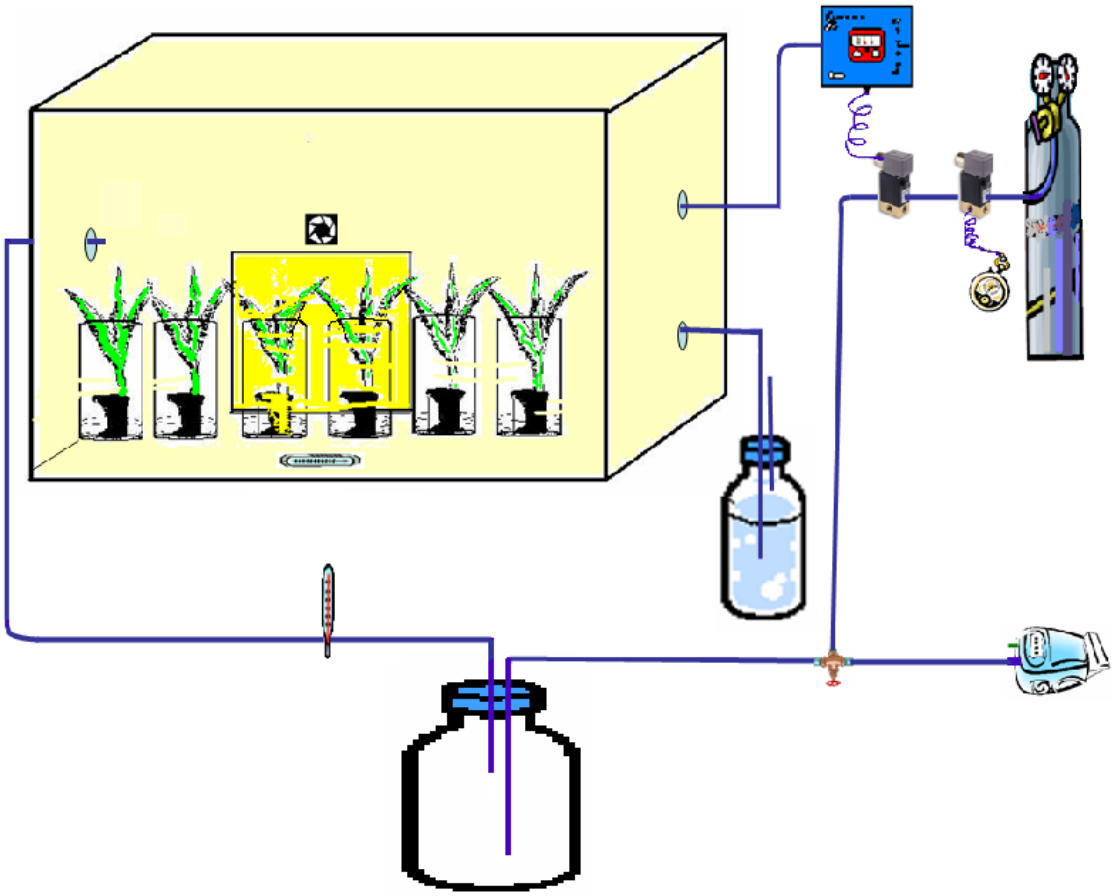

Figure 4. A CO_2 culture atmosphere enrichment system built for coconut. The chamber was made of transparent acrylic sheet (6 mm thick, 1100 mm long, 500 mm wide and 400 mm high). Pure CO_2 was pumped and mixed with ambient air (in a 2 L Schott bottle) before being passed into the chamber using silicon tubing (4 mm inner diameter). The elevated CO_2 concentration (1600 μmol mol^{-1}) generated in the chamber was maintained using a CO_2-monitor.

Figure 5. An acclimatisation box (a) used to complete the acclimatisation step prior to the seedlings being planted out into soil in a screen house. A bigger box or a tent (b) could be used when handling many seedlings. The tent is equipped with an automatic misting system and ventilation fan.

Figure 6. The application of the new embryo culture technique to the mass production of the high value, mutant coconut Makapuno.