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RESEARCH ARTICLE

AN ENHANCED EMBRYO CULTURE METHODOLOGY FOR COCONUT (*COCOS NUCIFERA* L.)

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ABSTRACT

Improvements to the conventional embryo culture protocol have been initiated. These improvements, among several, include the use of a CO_2-enrichment step within a photoautotrophic culture system and the application of 100 µM of either α-naphthalene acetic acid (NAA) or indolebutyric acid (IBA) to promote rooting of seedling. To obtain uniform germination of embryos of different ages, abscisic acid (ABA) at 0.97-7.67 µM is added to the germination medium. ABA could be replaced with gibberellic acid (GA_3) at 10.00 µM when handling varieties that are difficult to germinate. Following the *in vitro* acclimatization using the CO_2 enrichment system, the hardening-off process *ex vitro* in the screenhouse is done by placing the seedlings inside the acclimatization box that provides high relative humidity and sufficient headspace for growth. Alternatively, a clear plastic covered tent could be used instead when dealing with large numbers of seedlings. By using this enhanced methodology on the embryos of normal coconuts the *in vitro* stage can now be reduced from 10 to 12, to 3 to 4 months and the success rate in transferring embryos to seedlings in the field was improved from 50 to 100%. The enhanced methodology needs to be further refined and applied to the different mutant coconut types that are found around the world.

Key Words: Carbon Dioxide-Atmosphere Enrichment System, Coconut, Embryo Culture, Euwens Y3 Medium, Germplasm.

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 one uses in breeding new varieties of a wide range of coconut germplasm that is available from around the world. However, due to a number of factors including natural disasters, insect pest like the coconut scale insect, drought and loss of coconut growing areas due to the expansion of urban housing developments, golf courses, recreational parks, cemeteries and factories, this valuable coconut germplasm is being lost rapidly. Low income, smallholder coconut (*Cocos nucifera* L.) farmers have been facing difficulties for decades due to the declining price of copra, the traditional cash product from this palm. These farmers are now looking towards new higher, value products from coconut to make their industry viable over the longer term. There are a number of elite coconut types that have either a soft and flavorsome endosperm which have high commercial value in the confectionary and ice cream industries and the aromatic types with a high demand in the beverage industry. The soft endosperm types popularly known as the "Makapuno" in the Philippines, Vietnam, and Thailand, "Kopyor" in Indonesia, "Garuk" in Papua New Guinea, or "Dikiri Pol" in Sri Lanka are naturally occurring mutants which cannot germinate in nature as their endosperm cannot support the germination of the embryo. In the propagation of this mutant type, the embryo has to be removed from the fruit and grown *in vitro* to produce a seedling. The aromatic types found in Thailand and Vietnam are also thought to be naturally occurring mutants which can only germinate in nature at a very low rate. The same kind of embryo culture procedure has to be used to propagate this type. The first attempt at the embryo culture of the "Makapuno" mutant coconut was by De Guzman in the Philippines in the early 1960s. Subsequent studies have led to the commercialization of this technique so it is now possible even in other countries like Thailand to mass produce seedlings of the "makapuno" and the aromatic types. The common issues encountered in these projects are the low rate of conversion of the isolated embryos to plantlets, and the duration of the protocol which can be as long as one year. These issues ultimately lead to high production costs for the elite seedlings, well above that possible for subsistence farmers.

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However, a recent collaborative project, funded by the Australian Centre for International Agricultural Research (ACIAR) has made some improvements to the standard culture protocol. 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) in 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.

In order for these conservation genebanks to be operational for the ICG network or for other coconut germplasm collection and establishment programs, a safe and reliable mechanism for collection, moving and re-establishing coconut germplasm is required. The collection and movement of whole coconuts is clearly impractical due to their large size, and the fact that transferring uncleansed nuts is phytosanitary unsafe. For these reasons the collection 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 nut from which they are isolated (Harries 1982) and carry no known diseases.

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, Magdalita *et al*. 2010 a & b). Such techniques are also useful for rescuing embryos from the high value coconut mutant types like "Makapuno" and "Kopyor" (Rillo and Paloma 1992b). The technique can also be used for *in vitro* selection for various whole-plant traits like drought tolerance; (Karunaratne *et al*. 1991) and for cryopreservation of coconut germplasm (Assy-Bah and Engelmann 1992, Sisunandar *et al*. 2005).

Today a standard, internationally recognized coconut embryo culture technique is being 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) large discrepancies have been noted between different laboratories and this makes the protocol inefficient and unreliable. It is thought that many aspects of the *in vitro*-grown plantlets' physiology are not optimal and this possibly 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 enhanced methodology 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 like Australia, Indonesia, Papua New Guinea, Philippines and Viet Nam, and working over a three-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". Thus, the main objective of this study is to develop an enhanced methodology for the following: i) isolation and transport of coconut embryos, ii) initiation of *in vitro* coconut embryo cultures, iii) maintenance of coconut embryo cultures until the seedlings produced are ready for establishment in soil, iv) acclimatization of the coconut seedlings produced, and v) establishment of the coconut seedlings in soil in a nursery.

MATERIALS AND METHODS

Harvesting nuts as sources of embryos for culture: The use of coconut embryos with an appropriate age of about 10 to 11 months after pollination is an important factor in order to obtain the highest possible rate of seedling production. At this stage of development, 10 to 11 month old embryos that are contained in in the nut that have an exocarp that is just starting to become brown in color were used (Figure 1A). The basic rule that can be applied during germplasm collection trips for coconut embryo culture is that the harvesting of embryos from a bunch can proceed if at least one nut has attained the brown color stage. Other practices that should be observed while collecting nuts to be used for embryo culture includes: selection for healthy trees and nuts with the best qualities such as well-filled nuts and free from diseases and pests. However, nuts should not be rejected if they do not have a water-splashing sound when shaken. Such a selection step is often practiced in the nursery when selecting nuts for germination but for selecting nuts to be used as sources of embryos, this practice should not apply. The nuts harvested at 10 to 11 months of age after pollination may not produce such a sound upon shaking because their cavities may be completely full of water.

The shortest possible time from harvesting of nuts to the isolation of the embryo and its inoculation onto a tissue culture medium is preferred to take advantage of embryo viability. When collecting coconut germplasm from remote areas, it is recommended to collect more embryos to about 20% more than what is needed. This is being done to compensate for the loss of viability of some of the embryos while being transported to the laboratory. While in transit, the embryos can be 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).

Equipment and facilities needed for embryo culture: A cork borer (no. 10 or bigger, Figure 1B) was used for the extraction of endosperm plug containing the embryo of the nut. The cork borer can be replaced with a sharpened metal pipe that is pushed into the endosperm using a wooden hammer, if the cork borer is not available.

Polycarbonate culture vessels (Figure 1C) are normally used as culture vessels for the germination step and early seedling maintenance until at least one unfolded leaf has been produced on the seedling (Figure 1C-a). Bigger vessels are then needed for the subsequent steps as the seedlings grow (Figure 1C-b). A double vessel system, produced by inverting a second culture vessel over the first, the vessels being joined at the point where their rims touch, is used for large seedlings (Figure 1C-c). Alternative to this culture vessel system, is one that uses an autoclavable clear polyethylene plastic bag placed over the top of the seedling as an extension of the culture vessel (Figure 1C-d).

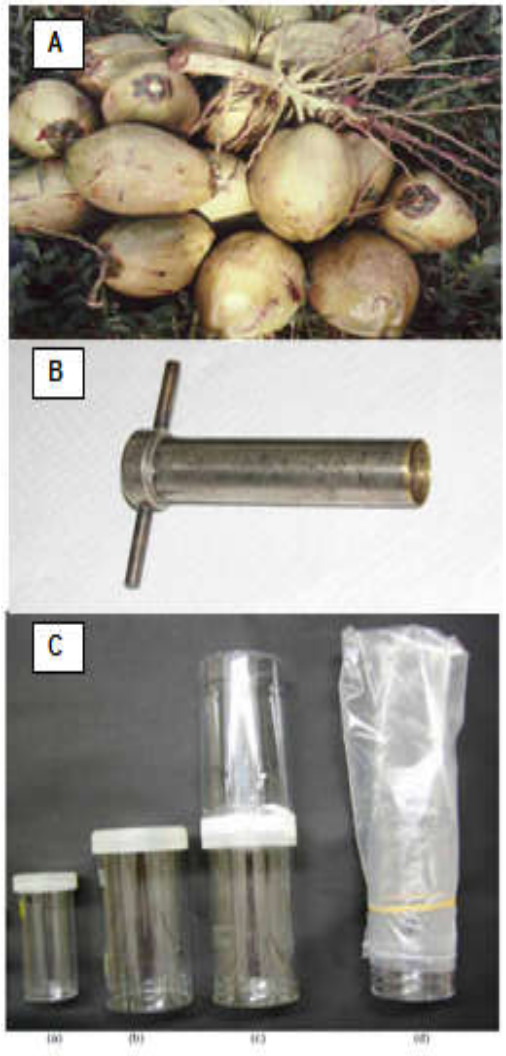

Figure 1. The ideal stage of development of coconuts for zygotic embryo isolation and culture is when several nuts in the bunch have just turned brown in color. At this stage, their age is estimated to be 10 to 11 months after pollination (A), borer used to remove the endosperm plug from the split nut (B), and the different types of polycarbonate culture vessels used in the embryo culture of coconut (C). Polycarbonate culture vessels (diameter 2.5 cm and height 8.0 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 autoclavable polyethylene plastic bag placed over the culture vessel (d).

A carbon dioxide (CO_2) atmosphere enrichment system (Figure 2) is used as an important improvement in the acclimatization of tissue cultured seedlings over the traditional method of continuing the incubation of the seedlings under an inverted clear plastic bag. The CO_2 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. An acclimatization box (Figure 3C) is used to complete the hardening-off process in a screen house prior to the seedling being planted out into a screen house. The acclimatization 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 holes per side). The four holes provide minimal exposure of the seedlings to natural screen house conditions, and therefore promote the hardening-off process of seedlings. 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 3D) could be used when acclimatizing large numbers of seedlings. The plastic cover on the box or the tent can be removed from time to time when implementing seedling management operations such as watering, fertilization or pest control.

Tissue culture media and reagents needed: The medium formulation developed for coconut embryo culture was based on Y3 minerals (Euwens 1976) with some modifications of vitamins and iron (Table 1).

Culture incubation conditions: Cultures were incubated at 28-30ºC under illuminated conditions (*ca*. 40 µmol $m^{-2}s^{-1}$) with a 14 hour light and 10 hour dark photoperiod. Red light enrichment (at *ca*. 660 nm) was added to promote seedling growth. A higher light intensity (*ca*. 90 µmol $m^{-2}s^{-1}$) should be used when growing the seedlings under photoautotrophic conditions.

Preparation of reagents and tissue culture media: Separate stock solutions (each 10 x the final concentration) of the six macronutrients 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) was placed in the test tube (25 x 150 mm) for culture initiation, solid medium (15 mL) was then used, followed by liquid medium (80 mL), in the last subculture in the bigger vessels. In all steps, the media should be kept stirred to distribute evenly the activated charcoal (acid washed, plant cell culture grade) during dispensing.

Embryo collection and extraction: The dehusked nuts were split with a bolo and a portion of the endosperm containing the embryo (endosperm cylinder) was removed using a surface sterilized cork borer. The endosperm cylinders were placed in a clean plastic beaker containing fresh coconut water, as an incubation medium. They were brought to the laboratory and washed using clean tap water and rinsed in ethanol (95%) for 30 s. The cylinders were then surface sterilized using fresh, full strength commercial bleach (normally containing 42 g L^{-1} sodium hyphochlorite) for 20 min. After this, they were rinsed three times with sterile water in a laminar air flow cabinet, to minimize the potential for recontamination.

The sterilized cylinders when transported to other laboratories, they were placed inside a sterile plastic bag with cotton wool moistened with sterile water. The bag was then placed inside a Styrofoam or in a thermally insulated box. To slow down degradation of the materials during transport, a small quantity of crushed ice to cool them was placed around the bags inside the box. The ice was replaced, as necessary, during the travel back to the laboratory. Alternatively, the samples were placed in a portable refrigerator connected to the car power supply. Another method of transport of the cylinders was used. The embryos were excised from the endosperm cylinders in the field and then transferred to the laboratory as soon as possible.

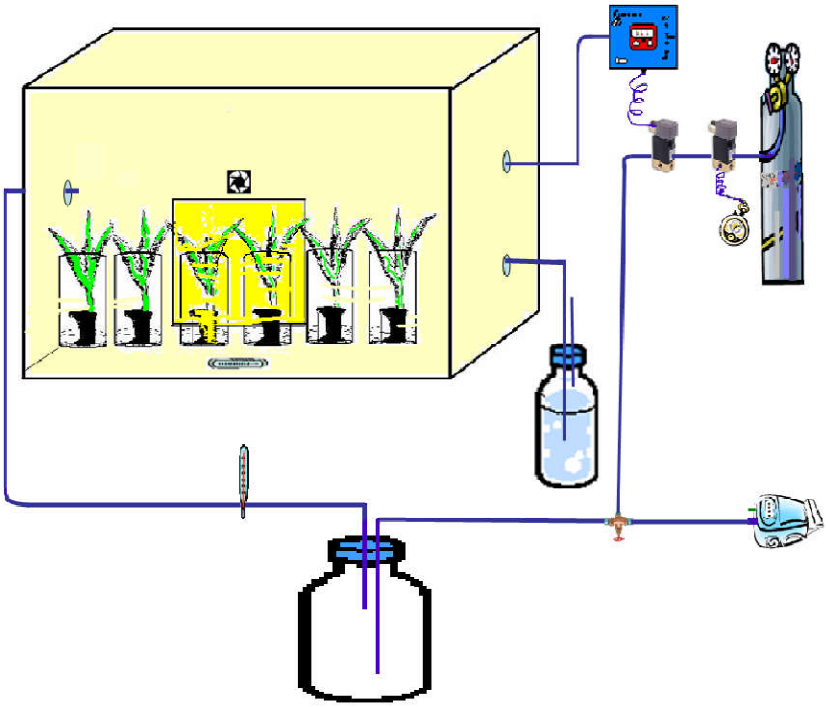

Figure 2. A CO_2 culture atmosphere enrichment system built for the acclimatization of tissue-cultured coconuts. The chamber was made of transparent acrylic sheet (6 mm thick, 1100 mm long, 500 mm wide and 400 mm high). Pure CO_2 was pumped and mixed with ambient air (in a 2 L Schott bottle) before being 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

Table 1. Media 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 orthophosphate	$NaH_2PO_4.2H_2O$	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	$NiCl.6H_2O$	0.240
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-panthothenate (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
Other components		
Abscisic acid (ABA)[2]	$C_{15}H_{20}O_4$	0.25
α-Naphthaleneacetic acid (NAA)[3]	$C_{12}H_{10}O_2$	18.60
Sucrose[4]	$C_6H_{22}O_{11}$	60.0, 45.0, 25.0
Agar (added only for the first and second subcultures[5]		7.0
Activated charcoal[6]		1.0
pH		5.6

[1]Iron chelate stock solutions were prepared in separate stock bottles.
[2]Filter-sterilized ABA was added to the germination medium to enhance germination.
[3]NAA was applied for one month only to promote root formation and development.
[4]Low grade sucrose can be used. Sucrose (60 g L^{-1}) was 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}) was 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.
UPLB – University of the Philippines Los Baños
ARC – Albay Research Center

Once isolated, the embryos are surface sterilized using fresh commercial bleach (10%, v/v for 1 min), 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 placed in a portable refrigerator. The samples were transported in the shortest possible time, not exceeding four days including the collecting and processing time.

Embryo culture

Stage 1 – The start up culture: In cases where endosperm cylinders or plugs were transported, upon arrival in the laboratory they were re-surface sterilized and rinsed in sterile water. Under aseptic condition, the embryos were excised from the endosperm cylinders and placed in sterile Petri dishes. In the case where the isolated embryos were transported, upon arrival in the laboratory they were transferred to a sterile Erlenmeyer flask and washed three times with sterile water, then transferred to sterile Petri dishes. The embryos were surface sterilized with 10% fresh, commercial bleach for 1 min and then rinsed with sterile distilled water three times. They were then transferred to sterile Petri dishes lined with filter paper to absorb any excess water.

The embryos were then inoculated individually into test tubes containing a liquid medium supplemented with abscisic acid (ABA, 0.25 mg L^{-1}). ABA is needed to get uniform germination particularly when using embryos of different ages. In addition, 10 µM gibberellic acid (GA_3) was added to replace ABA when germinating varieties with a low germination rate (Figure 3A). The embryos in liquid medium were then incubated in the dark for 1 month. After this time, the haustoria of the embryo were removed. The remaining portion was then transferred to a solid medium (same medium as above but containing 2% agar and with no ABA) and subcultured every month until shoots and roots developed. The germinated seedlings were then placed under illuminated conditions once they had attained at least 1 cm shoot growth. All the ungerminated seedlings were then discarded at the end of the 12 week incubation period.

Stage 2 – Early seedling growth (ie. up to the 1-leaf stage): The seedlings were sub-cultured into a liquid medium of the same formulation but containing 100 µM α-naphthalene acetic acid (NAA) to promote root formation and development (Figure 3B). After 1 month in the root-promoting medium, the seedlings were subcultured into a liquid culture medium without NAA. They were then subcultured every month into fresh medium until they have one unfolded leaf. At this stage, they were then transferred into either a conventional embryo culture growth condition or in a photoautotrophic environment if this apparatus is available.

Stage 3 – Further seedling growth (ie. up to the 3-leaf stage) under photoautotrophic culture using CO_2-enriched conditions: When the seedlings have produced at least one unfolded leaf, they were transferred into a phototrophic system and with CO_2-enriched conditions (Figure 2). The medium used in this system is the Y3 minerals with Fe-EDTA, but without sucrose. The seedlings especially the roots, were washed carefully in running tap water to remove the agar. They were placed into a Benlate™ fungicide solution (2 g L^{-1}) for 15 min, then they were transferred into 100 mL pots containing 10 g of an autoclaved vermiculite, or a coconut coir dust substrate, soaked with Y3 minerals. The pots were then placed into 500 mL culture vessels with 40 mL of a liquid medium containing Y3 minerals. The vessels with lids, were then placed into a CO_2-growth box. The box was then fogged with CO_2 (1600 ppm) for the illuminated portion of the photoperiod and during the dark portion with ambient air. The vessels were topped up with mineral solution and this was completely replaced every month.

Stage 4. Seedling acclimatization and pre-nursery care: The vessels were taken out of the laboratory and placed into a screen house for one week to start the hardening process. After one week in the screen house, the seedlings were removed from the vessels and washed with tap water. The seedlings were then dipped in Benlate™ fungicide solution (2 g L^{-1}) for 15 min and then planted individually into sterilized potting mix (garden soil and coconut coir dust in a 1:1 ratio) held in plastic bags and watered. The plants were placed in an acclimatization box (Figure 3C) where they are kept covered for three to four weeks or until the seedlings have recovered from *in vitro* conditions.

After this period, the seedlings were gradually exposed to the screen house conditions by partially lifting up portions of the plastic lid of the acclimatization box. The plants were then fully exposed to screen house conditions for one to two weeks. The plants were watered as required and a dilute foliar fertilizer solution was applied to their leaves each week. The seedlings were then maintained using the common coconut nursery practice conditions, including those of pest control, until they were ready for field planting.

When the seedlings have reached the three to four expanded leaf stage (normally after two to three months of growth under the CO_2-enriched conditions), they were ready to be transferred to the nursery. At the three to four expanded leaf stage, the CO_2 fogging was stopped and the seedlings were gradually exposed to ambient conditions, by partially opening the lid of the CO_2 box, over a two week period. The vessels were refilled with Y3 minerals when necessary. After two weeks, the seedlings were transferred to the screen house and planted into sterilized potting mix contained in black plastic bags.
A slow release fertilizer was added and a dilute foliar fertilizer solution was applied weekly for the first month. The seedlings were watered when needed and appropriate pest control measures were applied.

Stage 5 – Seedling transfer to the nursery: After three months of acclimatization under shade conditions, the seedlings were transferred to the nursery. The seedlings were planted into bigger polyethylene bags containing non-sterilized coconut coir dust mixed with soil. The seedlings were then placed in the nursery under partial shade and provided with the normal practices for coconut nursery management. After another three to five months, when the plants have four to six leaves, with at least one of them with leaflets, the seedlings were transferred to the field under normal planting conditions.

RESULTS AND DISCUSSION

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.

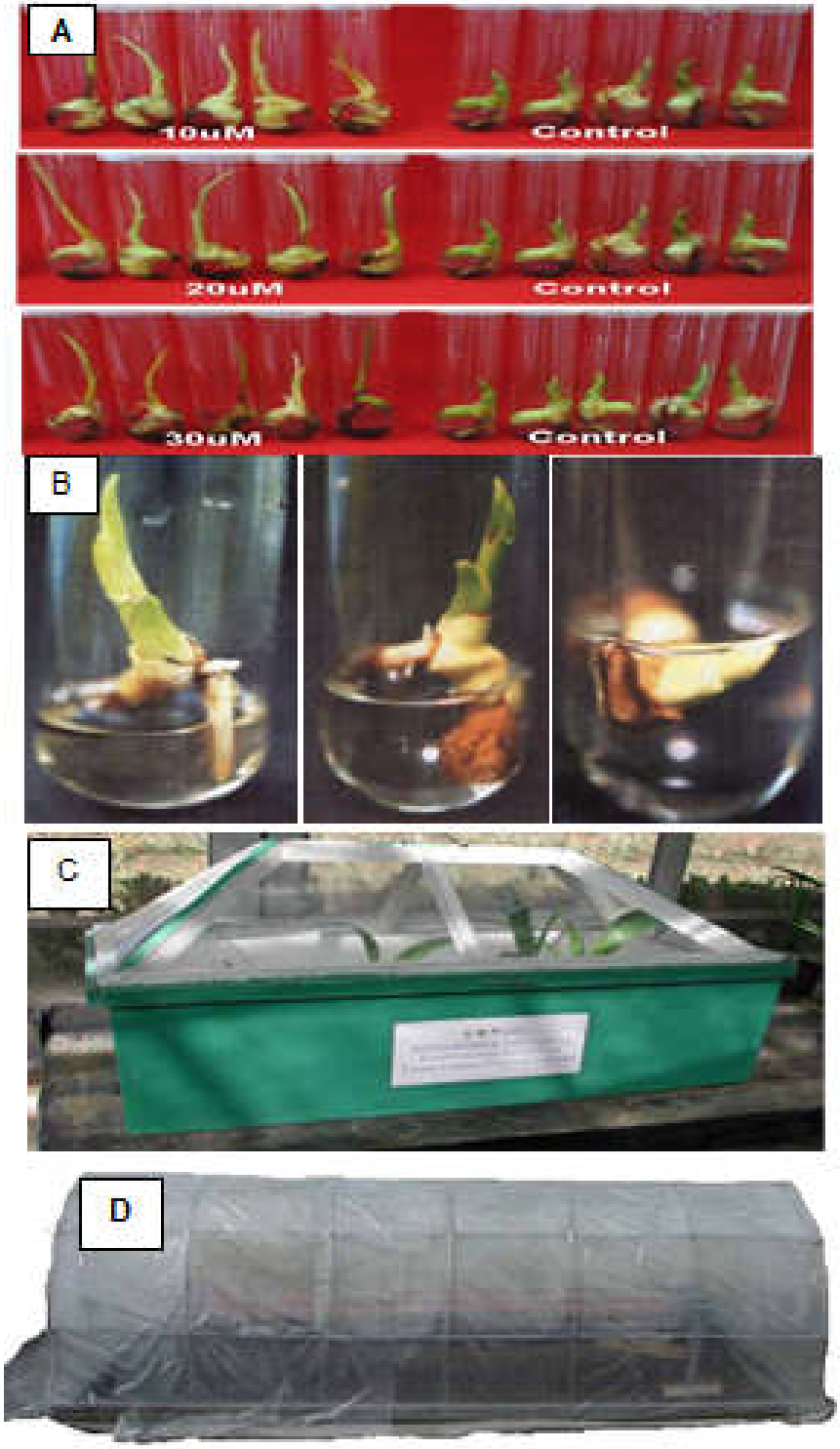

Figure 3. Shoot elongation of germinated 'Laguna Tall' coconut seedlings enhanced by GA_3 (10-30 µM) 10 weeks after inoculation on liquid Y3 medium (A). Root induction in germinated aromatic coconut embryos inoculated in liquid Y3 medium supplemented with IBA (2 mg L^{-1}) (B). An acclimatization box (C) used to complete the acclimatization step prior to the seedlings being planted out into soil in a screen house. A bigger box or a tent (D) could be used when handling many seedlings. The tent is equipped with an automatic misting system and ventilation fan

Embryo injury during the isolation process should be avoided. This is necessary to prevent wounds that may cause leakage of mineral ions and nutrients out of the embryos. Some of the embryos germinated within the first month after the start of culture. The germinated embryos were transferred onto the solid culture medium with care to ensure that 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. For various reasons, embryos and plantlets may develop abnormal features.

Figure 4. The embryo cultured seedlings resulting from the application of the new embryo culture technique to the mass production of the high value, mutant coconut 'Makapuno' at the Albay Research Center, Philippine Coconut Authority, Albay, Philippines

These may include hyper-hydrated tissues or stunted embryo growth. In most cases, these abnormalities were 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. 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. Additional 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 success rate using the CO_2-enrichment system in transferring embryos to seedlings in the field can be improved from 50 to 100%. This corroborated with the report of Xiao and Kozai (2004) that using the same system of acclimatization, percent survival of calla lilly (*Zantedeschia ellioltiana*) was increased to 95% while in the conventional photomixotrophic system, percent survival was only 60%.

The use of an atmospheric CO_2-enrichment system has been shown to significantly reduce the rate of seedling loss during the acclimatization steps. Similarly it has been shown that increasing the CO_2 concentration and light intensity but reducing relative humidity promoted the photoautotrophic growth of several woody species like acacia (*Acacia manglum*), mangosteen (*Garcinia mangostana*) and pine (*Pinus radiata*) (Kozai 2010, Kozai and Kubota 2001) including an ornamental plant like *Banksia* species (Godfrey and Cross 2005). The growth could be aided by the increased CO_2 during the photoperiod and light intensity that promoted photosynthesis (Kubota 2002).

In addition, the *in vitro* derived coconut seedlings while in this system were grown on a sugar-free medium in a ventilated vessel that is why CO_2 was supplied as carbon sucrose for the plant. The absence of sugar also reduces considerably the multiplication of microbes, thus avoiding the loss of plants due to microbial contamination. The reduction in relative humidity brought by the system could promote transpiration and thus uptake of minerals as well as improve the physiological and morphological characteristics of the plants *in vitro* (Kozai *et al*. 1995).

Furthermore, the use of a porous and light supporting material like vermiculite or coir dust instead of agar for the cultures being acclimatized using this system is advantageous since it has higher air porosity which gives higher dissolved oxygen concentration around the shoot base, thus promoting aeration contributing to growth of the plants. On the overall, the CO_2-enrichment system aids in the smooth transition from *in vitro* to *ex vitro* conditions that avoid malfunctioning of stomata, poor epicuticular wax development, low chlorophyll and slow growth rate of plants (Xiao and Kozai 2011).

Interestingly, culture of the seedlings under *in vitro* conditions were shortened from one year using the old hybrid EC approach to about four months using the new protocol. Similarly, the culture period for calla lilly (*Zantedeschia ellioltiana*) and china fir (*Cunninghamia lanceolata*) was reduced by 50% using the photoautotrophic system by CO_2 fogging (Xiao and Kozai 2004). When seedlings reach the one unfolded leaf stage, they were ready for photoautotrophic growth using the CO_2-enrichment system. These steps may only take two more months before the seedlings are ready for the acclimatization step and transfer to the screen house.

The photoautotrophic steps, however, require additional equipment, materials and some degree of operator skill. The extra cost however, may be compensated from savings in using less media and more seedlings were produced at the end of the procedure. Similarly, savings were recorded using the same CO_2-enrichment system in the mass propagation of calla lilly wherein the production cost was reduced to 40% while the initial cost of production per seedling was about 10% lower than the conventional photomixotropic system (Xiao and Kozai 2004). Abscisic acid (ABA) at 0.94 to 7.67 µM added to the germination medium provided uniform germination of embryos obtained from nuts of different ages. This finding conforms to the earlier results of Karun *et al*. (2002) that ABA promoted germination of 'West Coast Tall' coconut embryos. However, ABA could be replaced with GA_3 (10 µM) when culturing varieties that are difficult to germinate. GA_3 at 10-30 µM concentration promoted shoot elongation of germinated embryos (Figure 3A). This is consistent with the known role of GA_3 in stimulating cell division and stem elongation and its direct effect on hydrolysis of starch reserves in the endosperm resulting to energy production that stimulate germination and growth (Taiz and Zeiger 2006). This finding corroborated with the previous result that GA_3 promoted embryo germination of coconut (Pech y Ake *et al*. 2002, Weerakoon *et al*. 2002), papaya (Magdalita *et al*. 1996), kentia palm (Chin *et al*. 1988) and avocado (Skene and Barlass 1983).

A significant proportion of the work undertaken at the Albay Research Station of the Philippine Coconut Authority (PCA) (Figure 5) and at the University of Queensland (UQ) to develop the new protocol used 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^{-1} IBA instead of NAA (Table 1) was used to promote seedling root growth. Embryos were swollen one week after culture initiation and embryos began to germinate 2-3 weeks after culture initiation. Roots and shoots appeared 6-7 weeks after culture initiation of different coconut varieties. It has been known that auxins like IBA stimulate cell enlargement and promote root formation in many plants (Taiz and Zeiger 2006). Possibly it relaxes or loosens the cellulose fibrils causing the release of the crosslinking bonds which hold the microfibrils together causing the cell wall to swell like a balloon and become plastic to accommodate osmotic water intake, thus making the cells enlarge (Bidwell 1979).

Indolebutyric acid was thus applied through the whole subculture process until the first leaf formation. So far, there has been no comprehensive evaluation yet 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 similar response *in vitro* as the 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.

Summary and Conclusion

The smallholder coconut farmers have been experiencing difficulties for decades due to the decreasing prices of copra in the world market.

Due to this reason, farmers are now looking forward to new and higher value products from coconut to make their industry more viable. In nature, there are a number of elite coconut types that have either a soft, flavorsome endosperm like the "makapuno" or aromatic coconut water that can be used as a refreshing drink. The "makapuno" types have a high commercial value in the confectionary and ice cream industries while the aromatic types have a high demand in the beverage industry.

The soft endosperm types like "Makapuno" are naturally occurring mutants which cannot germinate in nature as their endosperm cannot support the germination of the embryo. To propagate this type plus the aromatic type, the embryo has to be removed from the fruit and grown *in vitro* to produce a seedling. The first attempt at the embryo culture of these mutant coconut types like 'Makapuno" was by De Guzman in the Philippines in the early 1960s. Succeeding studies have led to the commercialization of this technique so it is now possible to mass produce seedlings of the "Makapuno" in the Philippines and in other countries like Thailand and Vietnam.

The most common constraints in these works are the low rate of conversion of the isolated embryos to plantlets and the duration of the protocol to produce seedlings ready for potting-out which can be as long as one year. These ultimately lead to high production costs for the elite seedlings, well above that possible for subsistence farmers. A recent collaborative project, funded by the Australian Centre for International Agricultural Research (ACIAR) has made some enhancements to the standard embryo culture protocol namely the use of a CO_2-enrichment step within a photoautotrophic culture system, application of 100 µM of either NAA or IBA to promote seedling rooting, incorporation of ABA at 0.25 mg L^{-1} to the germination medium to obtain uniform germination of embryos of different ages and, the use of GA_3 (10 µM) instead of ABA when culturing varieties that are difficult to germinate. By using this protocol on the embryos of normal coconuts, the *in vitro* stage can now be reduced from 10 to 12, to 3 to 4 months and the success rate in transferring embryos to seedlings in the field can be improved from 50 to 100%. It is recommended that this enhanced protocol be used for routine embryo culture of coconut.

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