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Effects of medium replenishment and acclimatization techniques on growth and survival of embryo cultured coconut seedlings

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The effect of different media replenishment or *in vitro* irrigation techniques on seedling growth of 'Laguna Tall' coconut seedlings *in vitro* was studied. Likewise, the effect of *in vitro* acclimatization techniques using polyethylene glycol and polyvinylpyrolidone on growth and survival of coconut seedlings after acclimatization was investigated. In addition, the effect of three *ex vitro* acclimatization techniques and potting-out at two different stages of seedling growth on the survival and growth of coconut seedlings was studied. Replenishment of the medium or *in vitro* irrigation every 60 days and regular subculture every 30-40 days significantly promoted plant height and development of secondary roots. The two *in vitro* irrigation techniques significantly decreased the percent contamination of cultures. Polyethylene glycol (PEG, 10 and 20 mg L^{-1}) and polyvinylpyrolidone (PVP, 10 and 20 mg L^{-1}) significantly reduced the number of secondary roots and expanded leaves *in vitro* and the plant height and leaf production *ex vitro*. Acclimatization using the plastic tent and the wooden box humidity chamber gave higher seedling survival than the misting method. Furthermore, potting-out at two different stages of seedling development (i.e. two- and three-leaf stage) did not significantly affect growth and survival of seedlings after acclimatization.

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Email Address: pmmagdalita@yahoo.com
Submitted: April 12, 2010
Revised: June 9, 2010
Accepted: June 20, 2010
Published: July 16, 2010
Editor-in-charge: Gisela P. Padilla - Concepcion

KEY WORDS

acclimatization, coconut, embryo culture, i*n vitro* irrigation, media replenishment, polyethylene glycol, polyvinylpyrolidone

INTRODUCTION

The coconut palm (*Cocos nucifera* L.), popularly known as the tree of life, is one of the most important sources of oil on an industrial scale, and a good cash crop for at least 3.5 M smallholders in the Philippines. There are over 300 million coconut palms occupying some 3.3 M hectares of the country's 12 M farmlands, thus they dominate the landscape in around 80% of the provinces (DA-BAS 2009). The abundance of coconut palms makes the Philippines the top exporter of coconut in the world, although Indonesia is the top producer.

While some Filipinos called the coconut as the lazy man's crop because some farmers do nothing else except wait for its regular harvest every 45 days, still many regard it as the most important tropical palm as a source of oil. In 2008, the Philippines was the world's biggest exporter of coconut oil wherein 1 M tons are being exported yearly (Mañalac 2008). Likewise, the country has been producing a large amount of virgin coconut oil both for local and export markets (PCA 2006).

Despite this, coconut producers are experiencing low, and ever declining yields. The reason for this is that coconut production is constrained by a number of problems like the pest *Brontispa longissima*, poor nutrition due to lack of fertilizers, destructive typhoons, lack of good quality planting materials and aging palms that were planted many years ago using unselected types. The old plantations need to be replanted with new, high-yielding and disease resistant varieties. For this reason, the Philippine Coconut Authority (PCA) has been active in implementing a national program on planting and replanting of coconuts and fertilization is an important part of it. However, replacement of the old palms with new ones is constrained by lack of planting materials.

The limited number of good planting materials using whole nuts is the main constraint for the multiplication of new varieties to be used for massive replanting. A practical solution to this problem is the embryo culture of zygotic embryos giving rise to whole seedlings or vegetative multiplication using inflorescence tissues via somatic embryogenesis of superior varieties. This strategy provides a solution for the production of uniform planting materials.

Zygotic embryo culture of coconut has been used for rescuing embryos of high value coconut mutants, e.g. "makapuno", which have a jelly-like, endosperm tissue, and establishing seedlings from them (De Guzman and Del Rosario 1974; Cedo 2002, 2005; Assy-Bah 1986; Rillo and Paloma 1992a). In addition, the technique can 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; Malaurie et al. 2002; Sisunandar et al. 2005).

In vitro culture of coconut is found to be influenced by some factors. Polyethylene glycol (PEG), an osmotically active agent, is found to reduce the osmotic potential of the liquid medium that could induce the development of more adventitious roots needed for growth and development of tissue cultured plants. This chemical compound has been used in the tissue culture of grapes (Dami and Hughes 1997). Another compound polyvinylpyrolidone (PVP), has been reported to absorb phenolics through hydrogen bonding, thus preventing tissue oxidation and browning, and hence avoiding tissue damage (George and Sherrington 1984). When supplemented into the basal medium, PVP induces the development of good root system, e.g., in oak (*Quercus robur* L.) microcuttings (Malà et al. 2000). *Ex vitro* acclimatization using the plastic tent method and with misting has been shown effective for hardening coconut seedlings (Rillo and Paloma 1992b) and promoting 85-100% seedling survival (Cedo 2005). Orense et al. (2006) also observed that the use of a communal humidity tent for *ex vitro* establishment of embryo cultured coconuts resulted in 100% survival.

While it has been reported that specific *ex vitro* acclimatization techniques, medium replenishment and PEG-mediated acclimatization influenced seedling recovery of "makapuno" coconuts (Cedo 2005, Rivera and Santos 2003, Orense et al. 1995, Rillo and Paloma 1992b), the use of these techniques for normal embryos of 'Laguna Tall' coconut has not yet been investigated. This study aims to investigate the effect of the following: i) medium replenishment or *in vitro* irrigation on growth and contamination of cultures, ii) chemical-supplementation (i.e., PEG and PVP) on the *in vitro* growth and *ex vitro* survival of seedlings, iii) three acclimatization techniques on growth and seedling survival *ex vitro*, and iv) potting-out at two different stages of seedling growth on the *ex vitro* survival and growth of coconut seedlings.

MATERIALS AND METHODS

Collection and preparation of coconut embryo explants

The source of materials used for embryo culture was 10 to 11 months old coconuts obtained from a coconut farm in San Pablo City, Laguna, Philippines. The nuts were split open and the embryo plugs or cylinders were extracted using a stainless steel cork borer (size no. 10). The embryo plugs or cylinders were collected in a sterile plastic beaker containing sterile distilled water and then transferred to a sterile plastic bag before they were taken to the laboratory for disinfection. Initial sterilization of the embryo cylinders was done by washing with tap water three times followed by washing with 95% (v/v) ethanol for five minutes, and rinsing twice with sterile tap water. Disinfection of embryo plugs followed using pure commercial bleach for 20 minutes, and rinsed five times with sterile tap water (Rillo 1995). The embryos were isolated from the cylinders using a pair of sterile forceps and scalpel inside a sterile laminar flow cabinet, and collected in a sterile Erlenmeyer flask. All the isolated embryos were soaked in 10% (v/v) bleach for five minutes, and rinsed five times with sterile distilled water (Rillo 1995). In general, the embryos were germinated using the Y3 liquid medium (Eeuwens 1976) placed in 25 x 150 mm test tubes covered with plastic caps. Sucrose (6.0%, w/v) plus activated charcoal (0.1%, w/v) were added to the culture medium. The pH of the medium was adjusted to 5.7 using 0.1 N NaOH or HCl before autoclaving at 120ºC at 15 psi for 20 minutes. They were allowed to grow for four weeks, and then sub-cultured in semi-solid medium (0.35%, w/v Bacto-agar) with reduced sucrose (4.5%, w/v) and placed in 125 mL volume polycarbonate tubes for another four weeks. After this period, the germinated seedlings were grown for 16 weeks, subcultured into 400 mL volume bottles containing 100 mL liquid medium. All cultures were incubated at 25±1 ºC under 16 hours light (120 $\mu mol\ m^{-2}s^{-1}$) and eight hours darkness. The germinated seedlings were subcultured into the desired fresh medium on a regular basis. For the medium replenishment experiments, the previous medium was decanted every 60 days for four months and replaced with fresh medium. One batch of embryos was inoculated in the same medium and germinated for two months then cultured on the same medium supplemented with different concentrations of PEG and PVP and incubated for two months.

Embryos used as control were inoculated in the same medium devoid of PEG and PVP. Another batch of embryos was germinated for two months in GA_3-supplemented (30 µM) liquid Y3 medium and the resulting seedlings were used for the experiments on medium replenishment or *in vitro* irrigation for four months and *ex vitro* acclimatization.

Effect of different medium replenishment techniques on seedling growth *in vitro* and percent contamination of cultures

The effect of different medium replenishment techniques on *in vitro* growth was tested on two-month old pre-germinated coconut seedlings that were subcultured regularly during the first two months. Three treatments, namely: i) regular subculture every 30-40 days for four months with four subcultures, ii) *in vitro* irrigation every 60 days for four months with two times *in vitro* irrigation, and iii) *in vitro* irrigation every 60 days for four months with two times *in vitro irrigation* with coir fiber support, were used. Treatment 1 involves the transferring of the cultures onto fresh Y3 liquid medium every 30-40 days, while treatment 2 involves the removal of the old medium and replenishment with fresh Y3 medium on the same culture vessel every 60 days. Treatment 3 is the same as Treatment 2 except that the bottom of the culture vessel is overlaid with sterilized coir fiber as support to test if it will promote the growth of seedlings. The growth response *in vitro* including plant height, number of expanded leaves, number of secondary roots and percent contamination were assessed.

Effect of polyethylene glycol (PEG)- and polyvinylpyrolidone (PVP)-supplemented medium on seedling growth

The effect of polyethylene glycol (PEG) and polyvinylpyrolidone (PVP) on the *in vitro* and *ex vitro* growth of coconut seedlings was studied using two-month old pre-germinated seedlings. Three concentrations of PEG and PVP (0, 10, 20 mg L^{-1}) were used as treatments. These different concentrations were added independently into the Y3 liquid medium. Pre-germinated seedlings on Y3 liquid medium (two months old) and further incubated in the PEG- and PVP-supplemented medium for eight weeks were assessed for number of expanded leaves, and number of secondary roots. The seedlings were then acclimatized *ex vitro* using the plastic tent method. Assessment of selected growth parameters such as plant height, number of expanded leaves and percent survival was done one month after acclimatization.

Effect of *ex vitro* acclimatization techniques on seedling growth and survival

The effect of 3 different *ex vitro* acclimatization techniques was tested using 3-month-old coconut seedlings at the three-leaf stage. The cultures while still inside the vessels were taken out of the laboratory and placed in a bench inside the greenhouse for one week under partial shade provided by a black net, afterwhich, they were taken out of the culture vessels and the roots were washed thoroughly in running tap water. The roots were dipped in Benlate™ fungicide solution (2 g L^{-1}) for 15 min before planting in sterilized potting mixture composed of garden soil and coir dust (1:1, w/w) in 12 cm diameter plastic pots. Three treatments, namely: acclimatization by misting, use of plastic tent, and use of wooden box humidity chamber were used. The seedlings were acclimatized in each treatment for 1 month before growth parameters such as plant height, number of expanded leaves, girth width, leaf length and leaf width were taken. The percent survival of seedlings was also assessed.

Effect of potting-out at different stages of seedling development on growth and survival

The effect of potting-out at two different stages of seedling development on the growth and survival of *in vitro* grown coconut seedlings after acclimatization for one month using the wooden box humidity chamber was assessed. The seedlings were prepared for *ex vitro* acclimatization following the procedures mentioned above. Two treatments using two- and three-leaf stages of seedling development were used for acclimatization. Each treatment was replicated three times with 10 samples per treatment. Growth parameters such as plant height, number of expanded leaves, girth width, leaf length and leaf width were taken. Percent survival of seedlings was also assessed.

Statistical design and analyses

All experiments were conducted in completely randomized design (CRD). All treatments were replicated three times with 10 samples per treatment giving a total of 30 samples. The data gathered, including plant height, number of expanded leaves, number of secondary roots, girth width, leaf length, leaf width, percent contamination and seedling survival, were subjected to analysis of variance (ANOVA). Significant differences between treatment means were detected using least significant difference (LSD). The t-test was used to test the difference between treatment means for growth parameters (plant height, number of expanded leaves, girth width, leaf length and leaf width) and seedling survival after acclimatization of two- and three-leaf stage coconut seedlings. The SAS Program version 8.0 (SAS System 1985; Cary, North Carolina) was used for the analysis of the data gathered.

RESULTS AND DISCUSSION

Effect of different medium replenishment techniques on seedling growth and contamination of cultures

The three different techniques for medium replenishments of coconut seedlings significantly affected the plant height, number of expanded leaves, number of secondary roots and percent contamination of cultures (Table 1). *In vitro* irrigation of cultures after 60 days (two times *in vitro irrigation*) with no coir fiber support significantly promoted the number of expanded leaves. The plant height and the number of secondary roots were also promoted but not significantly different from regular subculture after 30-40 days. In contrast, *in vitro* irrigation after 60 days of culture (two times *in vitro irrigation*) and with dried coir fiber support did not promote plant height,

Table 1. Growth response of pre-germinated two-month old 'Laguna Tall' coconut seedlings after four months to medium replenishment or *in vitro* irrigation and regular subculture

Techniques for medium replenishment	Plant height (cm)	Expanded leaves	Secondary roots	Contamination (%)
Regular subculture every 30-40 days	17.2 [a]	2.1 [b]	2.6 [a]	58.0 [a]
Medium replenishment or *in vitro* irrigation after 60 days	18.1 [a]	2.7 [a]	2.5 [a]	21.0 [b]
Medium replenishment or *in vitro* irrigation after 60 days with coir fiber support	14.3 [b]	1.8 [b]	1.2 [b]	22.0 [b]

Means with the same letter superscript are not significantly different at 5% level by LSD

Table 2. Growth response and *ex vitro* survival of 'Laguna Tall' coconut seedlings treated with different concentrations of polyethylene glycol (PEG) and polyvinylpyrolidone (PVP)

PEG (mg L^{-1})	*In vitro* growth response		*Ex vitro* growth response after one month		
	Expanded leaves	Secondary roots	Plant height (mm)	Expanded leaves	Survival (%)
0	1.68 [a]	17.0 [a]	34.1 [a]	3.4 [a]	88.0 [a]
10	0.49 [b]	2.7 [b]	11.5 [b]	2.2 [b]	92.0 [a]
20	0.18 [b]	5.3 [b]	12.3 [b]	1.6 [b]	74.0 [a]
PVP (mg L^{-1})					
0	1.71 [a]	17.0 [a]	34.2 [a]	3.5 [a]	81.0 [a]
10	0.60 [b]	6.0 [b]	15.2 [b]	2.9 [b]	82.0 [a]
20	0.90 [b]	7.0 [b]	15.1 [b]	1.9 [b]	83.0 [a]

Means with the same letter superscript are not significantly different at 5% level by LSD

number of expanded leaves and number of secondary roots. This result suggests that the dried coir fiber did not support the growth of coconut seedlings *in vitro*. Coir fiber can be onsidered a suppressive substrate (Hyder et al. 2009) that contains tannins, glucoronic acid and uronic acid that makes it acidic which is possibly inhibitory to growth of coconut seedlings. This further suggests that *in vitro* irrigation can support the growth and development of germinated coconut seedlings. The growth accumulation for four months while they are under *in vitro* irrigation is possibly promoted by the remaining minerals still present in the old culture medium even after 30 days, and the new and enriched culture medium that was irrigated into the culture vessel after 60 days. The present result corroborated with the previous findings of Cedo (2002) that in the original medium, not all nutrients were utilized by the germinating seedling. Significant amounts of nitrogen, phosphorus and zinc were absorbed but other elements like potassium, calcium, iron, manganese, and copper were not absorbed; hence, they serve as sources of nutrients in addition to the new medium irrigated into the culture.

Furthermore, the percent contamination of cultures was also significantly affected (Table 1). It was significantly reduced in cultures that were irrigated after 60 days compared to those that were subcultured regularly after 30-40 days, which had very high contamination. This reduced contamination is possibly due to the irrigation technique that was done because there is minimal exposure of the cultured seedlings to possible contaminants from outside the vessel. For big cultures like the coconut seedling, it is very practical to allow them to remain inside the culture vessel and just replenish the medium to minimize the chances of contamination.

Table 3. Growth response and survival of three-leaf stage (three months old) embryo culture-derived 'Laguna Tall' coconut seedlings acclimatized *ex vitro* for one month using three different techniques

Techniques for *ex-vitro* acclimatization	Plant height (mm)^{ns}	Expanded leaves^{ns}	Girth width (mm)^{ns}	Leaf length (mm)^{ns}	Leaf width (mm)^{ns}	Survival (%)
Misting	75.0	2.1	1.1	50.0	2.8	62.5 ^{b}
Plastic tent	78.0	2.2	1.2	57.0	3.0	81.7 ^{a}
Wooden box chamber	80.0	2.3	1.3	60.0	4.0	82.2 ^{a}

Means with the same letter superscript are not significantly different at 5% level by LSD
ns = not significantly different

Table 4. Growth response and survival of two- (two months after explanting) and three-leaf stage (three months after explanting) embryo culture-derived 'Laguna Tall' coconut seedlings acclimatized *ex vitro* for one month using the wooden box humidity chamber

Stage of seedling growth	Plant height (mm)^{ns}	Expanded leaves^{ns}	Girth width (mm)^{ns}	Leaf length (mm)^{ns}	Leaf width (mm)^{ns}	Survival (%)^{ns}
Two-leaf	60.0	2.3	1.7	57.0	9.0	83.5
Three-leaf	67.0	2.4	1.4	60.0	7.0	84.2

ns = not significantly different at 5% level by t-test

Effect of polyethylene glycol-supplemented medium on seedling growth

Compared to the control, PEG concentrations at 10 and 20 mg L^{-1} inhibited significantly the number of expanded leaves, and the number of secondary roots (Table 2). These results corroborated the previous report that PEG is inhibitory to growth of germinating coconut embryos (Pech y Ake et al. 2002). This inhibition of growth and morphogenesis may be caused by the high osmotic potential of the culture medium due to PEG and high sucrose concentration (6.0%, w/v) placed in the medium. This also caused the formation of suberised tissue surfaces, hence limiting nutrient absorption by the plant. PEG has been reported to reduce the formation of apogamous buds giving rise to leaf formation from the prothalli of the bracken fern (*Pteridium aquilinum*) (Whittier and Steeves 1960), and the reduction on the rate of callus growth of *Fraxinus pennsylvanica* stem sections (Doley and Leyton 1970).

These results are contrary to the previous report that PEG can decrease the osmotic potential of the liquid medium, thereby allowing the plant to absorb more nutrients. Thus, there is enhanced development of more adventitious roots as has been observed in the *in vitro* hardening of 'Valiant' grape (*Vitis vinifera* L.) (Dami and Hughes 1997). The difference may be due to the genotypic response to PEG of the species used, suggesting that the effect of PEG on growth promotion could be species- or genotype-dependent.

The different PEG concentrations also decreased the growth *ex vitro* in terms of plant height and the number of expanded leaves. This decrease in growth is possibly a carry-over of the inhibited growth observed while the seedlings are still growing *in vitro*. In addition, the different PEG concentrations had no significant effect on the survival of acclimatized seedlings *ex vitro*. This contrasts with the result of Orense et al. (1995) who noted a decrease in survival of PEG-treated embryo cultured seedlings. The difference could be due to the PEG concentrations and the kind of embryos ("makapuno" vs. normal) used in the two separate studies. Overall, the inhibited growth of coconut seedlings both *in vitro* and *ex vitro*, and the insignificant surviving capacity of seedlings in the different treatments, imply that PEG is not necessary for promoting growth and *ex vitro* survival of coconut seedlings.

Effect of polyvinylpyrolidone-supplemented medium on seedling growth

Polyvinylpyrolidone (PVP) concentrations of 10 and 20 mg L^{-1} significantly inhibited the growth of 'Laguna Tall' coconut seedlings *in vitro* in terms of number of expanded leaves and number of secondary roots in comparison with the control (Table 2). The present finding is in contrast with the previous report of Malà et al. (2000) indicating that PVP induced the development

Figure 1. Acclimatization *ex vitro* of three-leaf stage (three months old) coconut seedlings using the plastic tent method (A), and the wooden box humidity chamber provided with plastic cover (B). Seedlings acclimatized inside the wooden box humidity chamber had better quality leaves that are well formed and shinier than those acclimatized using the plastic tent method.

of root system in oak (*Quercus robur* L.) microcuttings.

The same PVP concentrations significantly reduced the plant height and the number of expanded leaves *ex vitro*. However, the various PVP concentrations did not affect the percent survival of seedlings after *ex vitro* acclimatization. The result suggests that PVP is not necessary for coconut tissue culture. Corollary to this finding, Damasco (2002) indicated that PVP has only minimal effect on the *in vitro* growth and *ex vitro* survival of coconut seedlings.

Effect of three *ex vitro* acclimatization techniques on seedling growth and survival

The three techniques for *ex vitro* acclimatization using misting, plastic tent and wooden box humidity chamber did not significantly affect the plant height, number of expanded leaves, girth width, leaf length and leaf width after acclimatization of three-leaf stage 'Laguna Tall' coconut seedlings. However, percent survival of the seedlings was significant (Table 3). The plastic tent and the wooden box humidity chamber gave significantly higher percent survival of seedlings than misting. This result conforms to the findings of Orense et al. (2006) that communal humidity tent supported higher survival of embryo cultured seedlings *ex vitro*. However, this does not conform with the previous report of Cedo (2005) indicating that misting could increase seedling survival from 85 to 100%. The difference in the results obtained could be due to factors like the season of the year when the experiments were conducted and the prevailing weather conditions like temperature and the potting medium used. In the misting method, the leaves of the seedlings and the potting medium are always wet and this may have caused the rotting of the roots due to anoxia in the root zone and growth of pathogenic microorganisms. In the *ex vitro* acclimatization of rooted papaya nodal cultures, a high relative humidity (>90%) is needed to simulate the *in vitro* conditions during the first week of acclimatization, but the leaves remain

dry to avoid rotting of the plantlets (Drew 2003).

The result of the present study suggests that either the plastic tent or the wooden box humidity chamber method could be used for *ex vitro* acclimatization of coconut seedlings. The plastic tent is constantly touching the leaves of the seedlings, while misting constantly washes off the powdery substance (bloom) on the surface of the leaves that make them look dull, instead of being shiny because the bloom is removed on the upper surface of the leaves. The seedlings acclimatized using the wooden box humidity chamber with plastic cover had shinier leaves and better appearance than those acclimatized inside the plastic tent and the misting method (Figure 1). The better formation of the leaves is probably due to the ample space inside the chamber that provided proper aeration, allowing the leaves of the seedlings to grow freely inside the chamber. Similar benefits of papaya cultures have been reported using the perspex humidity cabinet being used in Australia for acclimatizing rooted nodal cultures (Drew 2003).

In contrast, in the plastic tent method, the narrow size of the plastic tent did not provide sufficient space for expansion of the leaves, limited air circulation and impeded the overall growth of the seedlings. In terms of practicality, the wooden box humidity chamber with plastic cover is more practical to use because it requires only minimal expense and would be more convenient to handle than the plastic tent and the misting technique. The use of the wooden box humidity chamber is also more advantageous than the plastic tent because its cover can be lifted gradually to expose the seedlings for them to adapt slowly to the ambient conditions in the greenhouse. In addition, the wooden box humidity chamber can be used repeatedly in acclimatizing *in vitro* derived coconut seedlings. However, because plastic bags are readily available, they have been used routinely for acclimatizing "makapuno" tissue cultured coconuts (De Guzman and Del Rosario 1974, Rillo 1995).

Effect of potting-out at different stages of seedling development on growth and survival

Ex vitro acclimatization for one month of coconut seedlings at the two- and three-leaf stage of development using the wooden box humidity chamber had no significant effect on growth and seedling survival (Table 4). The three-leaf stage seedlings are being used for acclimatization using the plastic tent method (Rillo and Paloma 1992b). While there are no significant differences in the growth response and survival of seedlings after *ex vitro* acclimatization for one month, the result suggests that potting-out and acclimatization can be done in either of the two stages of leaf development. Furthermore, the insignificant difference in seedling survival in the two treatments suggests that for practicality, acclimatization can be done as early as the two-leaf stage (two months old) using the wooden box humidity chamber to save time and resources while good quality seedlings can still be raised at the shortest possible time.

CONCLUSIONS

The different techniques for medium replenishment or *in vitro* irrigation significantly affected the plant height, leaf production, development of secondary roots of 'Laguna Tall' coconut seedlings, and contamination of cultures. The plant height and the development of secondary roots were significantly increased by *in vitro* irrigation of the cultures after 60 days, but were not significantly different from regular subculture every 30-40 days. The number of expanded leaves was significantly promoted using *in vitro* irrigation after 60 days. Medium replenishment significantly decreased contamination of cultures; hence, it is recommended for use in coconut tissue culture. Polyethylene glycol (PEG, 10 and 20 mg L^{-1}) and polyvinylpyrolidone (PVP, 10 and 20 mg L^{-1}) significantly reduced the number of secondary roots and expanded leaves *in vitro* and the plant height and leaf production *ex vitro*. Thus, PEG and PVP are not recommended for use in coconut embryo culture.

The three *ex vitro* acclimatization techniques, namely: misting, plastic tent and the wooden box humidity chamber, had no significant effect on seedling growth. However, seedling survival *ex vitro* is significantly affected by the acclimatization techniques. Seedlings acclimatized using the plastic tent and the wooden box humidity chamber had significantly higher survival than those seedlings acclimatized using the misting method. The wooden box humidity chamber method is recommended for *ex vitro* acclimatization because it allowed the development of better quality seedlings with well-formed and shiny leaves. Moreover, potting-out at the two different stages of seedling development (i.e. two- and three-leaf stage) had no significant effect on the growth and survival of seedlings after acclimatization. Hence, it is recommended that acclimatization of *in vitro* derived seedlings could be done either at the two- or three-leaf stage of development.

ACKNOWLEDGMENTS

The authors acknowledge the Australian Centre for International Agricultural Research (ACIAR) for the financial support, The project staff of the University of Queensland, Brisbane, Australia for the efficient project management and technical support, and the Crop Science Cluster-Institute of Plant Breeding, University of the Philippines Los Baños (UPLB) for the physical facilities. The assistance rendered to the authors by Ms. Leila S. Caymo, Ms. Lolita DC Valencia, Ms. Abigail May R. Oropesa, Mr Joseph C. Beredo, Mr. Nicasio T. Wagan and Mr. Bill Anderson is also acknowledged. The useful suggestions of the reviewers are highly appreciated.

CONFLICTS OF INTEREST

This study has no conflicts of interest with other researches on coconut tissue culture from the Philippine Coconut Authority and other units of UP Los Baños, rather it is complementary.

CONTRIBUTION OF INDIVIDUAL AUTHORS

Dr. PM Magdalita is the leader of the project entitled, "Acclimatization and Seedling Establishment *Ex Vitro* of Tissue Culture-Derived Coconut", from where this article was based. He wrote the article, while Dr. OP Damasco is the study leader of the project. Both authors performed the experiments for this study equally.

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