RESEARCH ARTICLE

Alternative Materials for Plumule Initiation and Callus Induction of Coconut (*Cocos nucifera* L.) cv. Laguna Tall

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Abstract

Coconut (*Cocos nucifera* L.) is an important Philippine export crop. Coconut yields in the Philippines are low owing to high genetic variability and old, senile trees. To provide better quality replacement planting materials, somatic embryogenesis was introduced in the country. It produces uniform, disease-free planting materials, in a high volume at a shorter time than seedling propagation. However, this modern technology requires expert skill, costly equipment, and specialized laboratory-grade materials. Hence, low-cost material options must be considered. Using Laguna Tall coconut embryos, the plumule initiation study was laid out in a two-factorial completely randomized design with sugar (sucrose, white table sugar) and duration (1, 3, and 5 d) as factors. Likewise, the callus induction experiments had culture vessel (45, 60, and 100 mL) and sealing materials (polypropylene sheets with rubber band, aluminum foil with masking tape) as factors. Data were analyzed using ANOVA with LSD for mean separation. Plumule growth was initiated to remove the need for microscopy in plumule excision prior to callus induction. For plumule initiation, embryo plugs were held for 3 d in liquid medium with sucrose resulting in plumule protrusion. Highest percentage of healthy explants was produced in 45-mL glass jars. Moreover, white calli were most abundant in cultures sealed with aluminum foil and masking tape. The combination of 45-mL glass jar and aluminum foil with masking tape was the only treatment that produced white calli at the second culture cycle. Relative cost analysis further revealed that 45-mL glass jars sealed with aluminum foil and masking tape was cheapest at PhP 7.32 compared to 100-mL jars with polypropylene sheets and rubber band at PhP 22.68. Results suggest that the use of 45-mL glass jars with the combination of aluminum foil and masking tape can be cheaper alternatives to conventional materials for coconut plantlet production.

Keywords: coconut • culture vessel • low cost • sealing material • somatic embryogenesis

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Introduction

Coconut palm (*Cocos nucifera* L.) is a major tropical plantation crop. The Philippines is the second largest coconut producer in the world next to Indonesia (PCA 2015; FAOSTAT 2017) and exports copra, coconut oil, copra meal, and other coconut products (Maierbrugger 2013). Furthermore, coconut fruits at 14.7 M tonnes from 347 million trees in 2018 (PSA 2017), remains an essential commodity for local and export markets and as a source of livelihood for 2.5 million coconut farmers (PCA 2020).

Among local coconut cultivars, Laguna Tall is most planted and used for producing ideal pure populations or for open pollination (Magat and Canja 2006). Although there is an abundance of coconut palms in the country, they are relatively old, and the coconut scale insect (*Aspidiotus* sp.) infestation (Ronda 2016) contributed to its drastic population decline along with typhoon uprooting (Abustan et al. 2016).

Seeds of coconut palms take a long time to develop into seedlings (Sukamto 1996) and have low success rate of growing into a mature plant (Harries 2012). There is low multiplication rate in using seeds since palms only produce 100 nuts annually, which puts pressure on seeds needed for propagation and for consumption (COGENT 2018). Also, palms grown through seeds allow genetic variations leading to non-uniform yield and varying responses to pests and diseases (COGENT 2018). These listed problems provide opportunity for vegetative propagation, which can produce uniform disease-free plantlets in a shorter time compared with seedling propagation. To mature, coconut fruits takes 11 to 14 months; and to produce seedling at a rate of 1 plant per seed, 7 to 9 months (Harries 2012). Using tissue culture methods, however, one embryo can become hundreds of uniform and disease-free seedlings at 270 d after culture (Lédo et al. 2019).

Almost every part of *C. nucifera* has been used as explants in tissue culture (Lédo et al. 2019). Among plant parts tested, callus formation and embryogenic capacity were better when zygotic tissues and plumules were used (Abustan et al. 2016; Ronda 2016; Chan et al. 1998) since juvenile tissues have numerous meristematic points, which make them more receptive to in vitro treatments (Bonga 1982). While plumules were found to be more successful for tissue culture, the size of the plumules makes it difficult to resolve without a microscope. To address this problem, cultured embryos in modified Eeuwens Y3 liquid medium with 6-benzylaminopurine (BAP), sucrose, and activated charcoal (AC) resulted in plumule initiation (Fernando et al. 2009), which removed the need for excision under a microscope.

While tissue culture is widely used in the industry, there are still gaps for improvement. One gap is on the culture vessel used as it affects shoot length, proliferation rate, and fresh weight of the plants produced (Fujiwara and Kozai 1995). Several types of culture vessels are used such as glass test tubes and petri dishes on induction stage, but larger vessels were used when transferring explants for multiplication and elongation (Ahloowalia et al. 2004). In commercial tissue culture laboratories in Davao City, for example, glass jars previously used for food are widely used to cut down on costs, but no peer-reviewed publications in the Philippines have evaluated their use. Another factor that contributes to the quality of cultured plants is the sealing material, which includes metal closures, polyurethane films, silicon closures, plastic covers, cotton fiber, and polypropylene films (Tanaka et al. 2005). Huang and Chen (2005) used polycarbonate plastic cover, which resulted in low gas exchange rates, while Zobayed (2000) used permeable cotton fiber,

which resulted to higher plantlet quality and less propagule loss during acclimation. In commercial tissue culture facilities abroad, transparent polypropylene films of about 1 mm thin or less have been used as a capping material (Mahlberg et al. 1980) for cultures requiring illumination. However, Sinta and Riyadi (2011) suggested aluminum foil as a preferable sealing material compared to plastic wrap or autoclavable plastic to prevent shunting of tissue growth of oil palm plantlets in vitro under low light intensity. No published local studies on comparison of sealing materials were found, but local commercial tissue culture facilities were observed to use polypropylene sheets or metal lids.

Furthermore, while tissue culture technology can be expedient for producing uniformly high-quality explants, it is very costly in terms of capital, labor, and energy (Ahloowalia et al. 2004). Several cost-cutting measures have been explored in many laboratories worldwide, including changes in growth medium composition, carbon source, and culture vessels. In the Philippines, one study is available on using table sugar as carbon source alternative in coconut micropropagation (Datta et al. 2017). This has provided an opportunity to explore low-cost alternatives for laboratory or analytical-grade materials for micropropagation of coconuts in the Philippines.

The establishment of a cost-efficient tissue culture methodology for producing coconut planting materials is important to the coconut industry. The availability of clean and healthy planting materials at any given time can greatly increase agricultural productivity and aid in national development.

Materials and Methods

Experiment Duration and Trials

The study was conducted at the Dr. Severo Bastian Jr. Plant Science Laboratory, College of Science and Mathematics Building, University of the Philippines Mindanao, Davao City, from January 2017 to February 2018. There was only one trial for the plumule initiation experiment and three trials for callus induction experiment. Trials were initially performed by a student who was assisted by the adviser in the first trial of the callus induction experiment then allowed to continue independently in the succeeding trials.

Sample Collection

A total of 250 Laguna Tall coconut fruits aged at 10 to 12 months old after pollination (Jayaraj et al. 2015) were purchased from the Philippine Coconut Authority (PCA)–Davao Research Center. The fruits were cored with a fabricated metal borer (1.3 × 2.0 cm) to obtain cylinders of endosperm containing embryos. Embryos were excised with sterile scalpel blade based on Ashburner et al. (1995), disinfected with 95% ethyl alcohol for 2 mins, and washed thrice with distilled water for 5 min each (Sukendah and Cedo 2005; Pérez-Núñez et al. 2006).

Plumule Initiation

A total of 45 embryo plugs were used for plumule initiation. To avoid the use of microscope in plumule excision, plumules in embryos were initiated in Eeuwens liquid basal medium (Eeuwens 1976) and 3% (w/v) of randomly selected UNIVAR™ sucrose based on Fernando et al. (2009). pH of the solution was adjusted dropwise to 5.8 using 0.1 M NaOH or 0.1 to 0.5 M HCl before adding 0.25% w/v Sigma-Aldrich acid-washed Activated Charcoal (AC) powder based on Fernando et al. (2009). The prepared media was dispensed evenly on 45-mL screw-capped jars at 10 mL each, autoclaved, then stored in a sterile environment prior to use. Laminar flow hood was first surface sterilized with 70% ethanol and ultraviolet light was turned on for at least 30 mins prior to use. Embryos were carefully removed from the disinfected plugs under the laminar flow hood using sterile scalpel and forceps. Embryos were then cultured in the liquid basal medium and kept under dark conditions at 28±1 °C until protruded plumules on one end of the embryo were seen.

Callus Induction

The first and second trials of callus induction experiment used 60 plumule explants while the third trial only used 48 explants due to limited resources. Callus induction medium used was composed of Eeuwens basal medium (Eeuwens

1976) with 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP), sugar, acid-washed activated charcoal, and agar to optimize mature zygotic embryo culture of coconut as recommended by Chan et al. (1998) and Perera et al. (2009). Prepared medium dispensed in culture vessels and sterilized at 15 psi for 15 min and were stored for about one week prior to culture as recommended by Ebert et al. (1993) to equilibrate growth regulators in AC (Sukamto 1996).

Plumular tissues were then excised in a laminar flow hood with a sterile scalpel and blade, cut into less than 1-mm pieces, and cultured in sterile culture vessels of various sizes (45, 60, and 100 mL) with 10 mL of callus induction media. Vessels were sealed with polypropylene sheets and rubber band or foil and masking tape. The cultures were then kept in dark conditions at 28±1 °C for at least 60 d or until embryogenic callus was induced. When calli transformed to calloids, excision was done and subcultured to the same callus induction media. All tissues were subcultured regularly according to Pérez-Núñez et al. (2006). Data on explant health and calli growth were collected prior to each subculture.

Data Gathered

Presence or absence of protruded plumules were recorded for the plumule initiation experiment. Morphological observations on the plumule explants for callus induction were done visually every two weeks to allow the cultures to grow relatively undisturbed. Color of explants and calli were monitored and visually rated based on Agaton et al. (1989), Armstrong et al. (1997), and Pérez-Núñez et al. (2006) with white, cream, yellow, green, light green, and brown as colors. Health of explants and calli were assessed every two weeks using devised classifications as healthy (absence of necrosis, disease or damage), necrotic (evident browning), or infected (fungal or bacterial contamination) based on Cassells (1991). Actual cost of culture vessels and sealing materials based on seller prices in 2017 were also recorded.

Culture Maintenance and Growth Conditions

Plumule cultures for initiation were suspended in liquid medium for 1 to 3 d while explants for callus induction were subcultured regularly every 30 d according to Pérez-Núñez et al. (2006). All cultures were stored in cardboard boxes to simulate dark conditions and room temperature was maintained at 28±1 °C.

Experimental Design and Analysis

The plumule initiation experiment was laid out in a factorial completely randomized design (CRD) with one embryo used as explant per replicate.

For the plumule initiation, there were two factors tested: sugar (distilled water [no sugar], sucrose, and Victorias® white table sugar) and duration (1, 3, and 5 d), with 5 replicates per treatment or a total of 45 embryo plugs used for the experiment.

For the callus induction experiment, culture vessel with different volumes (45, 60, and 100 mL) (Figure 1) and sealing material (polypropylene films with rubber band, and aluminum foil with masking tape) were used as factors; and there are at least ten replicates per treatment and a total of 168 explants used. Ten replicates in each of the six treatments and three trials for the entire experiment except for trial 3 with only eight replicates due to limited time and resources. The three trials were performed on different periods as follows: trial 1 from March to May 2017, trial 2 from June to August 2017, and trial 3 from December 2017 to February 2018.

For the plumule initiation and calli induction experiments, data were analyzed using analysis of

FIGURE 1 Culture vessels used in the callus induction experiment of *Cocos nucifera* L. cv. Laguna Tall in various sizes based on volume (left to right): 45, 60, and 100 mL.

variance and Fisher's least significant difference in SPSS (GPS-0939539-TEOA-F7156).

Results and Discussion

Plumule Initiation

Time to allow for greater plumule growth (Figure 2) prior to excision from the embryo is essential to facilitate its excision without the aid of a microscope. In the present study, results suggest that a three-day incubation in culture medium with or without sugar promoted plumule growth (Table 1). Aside from this period being shorter, it also required a lower sucrose level in the liquid basal medium for the plumule initiation of Laguna Tall coconuts. In Sri Lanka Tall coconuts, 6% (w/v) sucrose was used in MS medium for 15 to 17 d (Fernando et al. 2009). Sucrose is expected to result in increased plumule growth since its pure form induces near optimal rates of growth as a widespread transport molecule (Mello et al. 2001; Placide et al. 2012; Saad and Elshahed 2012). Sucrose has high solubility in water, electrically neutral, and is shown to have no inhibitory effects on majority of biochemical processes (Placide et al. 2012). Data suggests, however, that sterile distilled water, sucrose, or white table sugar have similar effects on plumule growth. Nonetheless, the low samples per replication in this study could be a reason for the non-significant differences between the two sugar sources used, indicating that white sugar can substitute for sucrose.

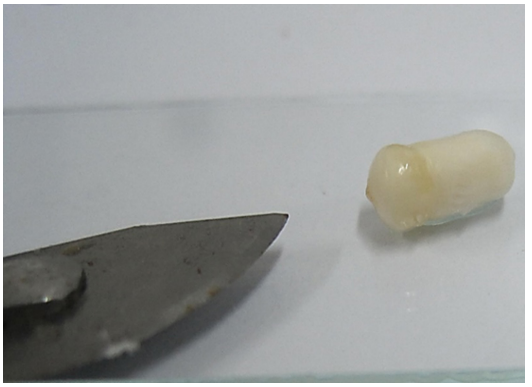

FIGURE 2 Coconut (*Cocos nucifera* L. cv. Laguna Tall) embryo plug with protruded plumule (arrow) 3 d after plumule initiation in liquid Eeuwens basal medium with sucrose.

TABLE 1 Mean (±SE) percentage of coconut (*Cocos nucifera* cv. Laguna Tall) embryos with protruded plumules as cultured for 1, 3, or 5 d in Eeuwens liquid medium supplemented with various sugars

Culture duration (days)	Embryos with protruded plumules (%)*
1	20.00^{b}±0.93
3	73.33^{a}±0.53
5	20.00^{c}±0.93

*Means within column with same letters are not significantly different at α = 0.05 (p-value = 0.00, N = 45).

Explant Growth

Prior to presence of calli, explant was observed to be initially white in color, eventually turning cream, an observation consistent with Haque et al. (1999). Throughout the development, the explants turn yellow in color and eventually produce calli or become brown due to necrosis. Necrosis is often associated with physiological age and failure of survival of explants (Krishna et al. 2008; Guo et al. 2010), as well as contamination (Modgil et al. 1999). Necrosis may be severe in some explants, causing inhibition or cessation of growth (Abdelwahd et al. 2008; Misra et al. 2010). Furthermore, inhibitory agents such as phenolics, tannins, or oxidized polyphenols are produced by plants (Preece and Compton 1991), causing necrosis in cultured tissues.

Vessel size had a significant influence on health of explants for trial 1 but not in other trials. Among the three sizes of culture vessels, highest percentage of healthy explants was observed in 45-mL vessels, which were statistically comparable to 65-mL vessels for trial 1 (Table 2). This suggests that smaller vessels can sustain healthier explants in the initial stages of callus initiation and is consistent with the recommendations of Ahloowalia et al. (2004) to use narrow-mouthed vessels. Results, however, contrasts with the findings of Adkins et al. (1993), which reported that wide-mouthed vessels were better for tissue culture for rice. The present results were also contrary to the study of

TABLE 2 Percentage of healthy *Cocos nucifera* L. cv. Laguna Tall explants under various culture vessel sizes after third week at second culture cycle for one trial

Vessel size (mL)	Mean percentage (±SD) of healthy explants (%)*
45	50.00±0.00[a]
60	43.75±7.07[a]
100	6.25±7.07[b]

*Healthy = color white with absence of bacterial or fungal growth; means within column with the same letters are not significantly different at α = 0.05 (p-value = 0.029). Trial N = 60; experiment N = 168.

TABLE 3 Percentage of healthy *Cocos nucifera* L. cv. Laguna Tall explants under various sealing materials after three weeks of culture at second culture cycle for one trial

Vessel size (mL)	Mean percentage (±SD) of healthy explants (%)	
	Polypropylene sheets with rubber band	Aluminum foil with masking tape
45	40	50
60	40	60
100	20	90
Mean (±SD)*	33.33±11.55	66.67±20.82

*Means within row are significantly different at α = 0.05 (p-value = 0.009); trial N = 60; experiment N = 168.

McClelland and Smith (1990), which used vessels from 200 to 350 mL as large vessels. The 100-mL large vessels, the largest among the vessels used in this study, did not differ from the explants cultured in 60-mL glass bottles. Adkins et al. (1993) found that large vessels provide a more diluted ethylene concentration and greater levels of oxygen in the environment surrounding the cultured tissues.

Sealing material on the culture vessels showed an effect on health of explants in trial 2. Cultures sealed with aluminum foil and masking tape showed higher percentage of healthy explants compared to polypropylene sheets with rubber band (Table 3). This finding was similar to the results of Sinta and Riyadi (2011), which recommended the use of aluminum foil over plastic sealing materials for oil palm plantlets. Air porosity and light of the sealing materials could influence the environment of the cultured tissues. Porous materials allow gas exchange to prevent ethylene build-up and reduce humidity (Lentini et al. 1988). Furthermore, porous materials help prevent oxidation of phenolic compounds that are released from the cut ends of the explants by polyphenolyloxidases (Mayer and Harel 1979) and peroxidases (Loomis and Battaile 1966; Vaughn and Duke 1984). Aluminum foil may have been able to provide the right amount of humidity and gas exchange necessary for the healthy growth of coconut tissues.

Callus Induction

Total callus count suggests that explants under foil and masking tape as sealing material produced more calli than sealed with polypropylene sheet and rubber band (Table 4). The transparency of polypropylene sheets hinders proper growth of cultures since light is not needed during this explant stage of development (Dodds and Roberts 1985). In a separate study, foil was found more effective over plastic wraps in maintaining culture temperature (Sinta and Riyadi 2011). It also lowers rates of hyperhydricity for improved plantlet quality at later stages in development (Chanemougasoundharam et al. 2004). It was further observed to act as an impermeable barrier to oxygen and water vapor, preventing gas exchange in cultures and lessening the occurrence of desiccation (Saccharaw and Griffin 2008). These properties of foil seem to be favorable for Laguna Tall plumules as more explants produced calli in culture vessels sealed with foil.

Among treatments, cultures with 45-mL volume produced highest percentage of explants with white calli during trial 1 (Figure 3, Table 5) but no significant differences were seen in trials 2 and 3. This result was contrary to Adkins et al. (1993) where culture growth was observed to be more ideal in larger vessels due to diluted ethylene concentration and larger oxygen availability. The stress on reduced oxygen availability brought about by the small vessel may have induced callus formation for coconut plumules since low oxygen availability also causes morphogenetic and physiological changes on tissues (Gould and Murashige 1985; Lai et al. 2005).

TABLE 4 Mean percentage of explants with callus formation for each treatment of *Cocos nucifera* L. cv. Laguna Tall explants after three weeks of culture at second culture cycle grown in vitro under various culture vessel and sealing material in three trials

Trial	Size by volume (mL)	Percentage of callused explants (%)		Mean(±SD)[NS*]
		Polypropylene sheet with rubber band	Aluminum foil with masking tape	
1	45	20.00	0.00	10.00±14.14
	60	10.00	10.00	10.00±0.00
	100	0.00	0.00	0.00
	Mean (±SD)[NS**]	10.00±10.00	3.33±5.77	
2	45	0.00	20.00	10.00±14.14
	60	0.00	0.00	0.00
	100	0.00	0.00	0.00
	Mean (±SD)[NS**]	0.00	6.67±11.55	
3	45	12.50	25.00	18.75±8.84
	60	12.50	0.00	6.25±8.84
	100	0.00	12.50	6.25±8.84
	Mean (±SD)[NS**]	8.33±7.22	12.50±12.50	

*Means within columns are not significantly different at α = 0.05. Data transformed using arc sine for normal distribution. Trial 1 N = 60, p-value = 0.347 (NS); trial 2 N = 60, p-value = 0.115 (NS); trial 3 N = 48, p-value = 0.435 (NS); experiment N = 168.
**Means of experiment trials are not significantly different at α = 0.05. Data transformed using arc sine for normal distribution. P-value = 0.481 (NS); experiment N = 168.

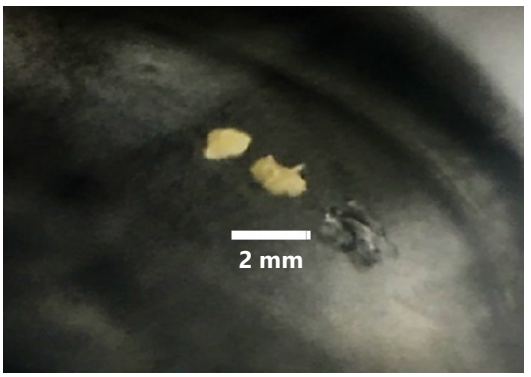

FIGURE 3 Calli growth from representative coconut (Cocos nucifera cv. Laguna Tall) plumule samples

TABLE 5 Mean percentage (±SD) of explants for each treatment producing white calli of *Cocos nucifera* L. cv. Laguna Tall explants after three weeks of culture at second culture cycle grown in vitro under different culture vessel size in one trial

Vessel size (mL)	Explants with white calli (%)*
45	25±21.21[a]
60	0[b]
100	0[b]

*Means within column with different letters are significantly different at α = 0.05 (p-value = 0.004). Trial N = 60; experiment N = 168.

Results suggest that a combination of 45-mL culture vessels and aluminum foil with masking tape produces the most ideal cultures with highest rate of healthy explants, which produce callus at third week of the second culture cycle.

Relative Costs

Tissue culture requires technical skills and laboratory or analytical-grade materials and equipment, which makes the method expensive (Lédo et al. 2019; Wijerathna 2015). In developing countries such as the Philippines, resources are not readily available if not expensive; hence, low-cost options for micropropagation must be considered (Datta et al. 2017). The combination of 45-mL bottles with aluminum foil and masking tape as sealing material was least expensive at PhP 7.32 compared to using bigger vessels and polypropylene sheets with rubber bands which reached triple that cost (Table 6). Traditional tissue culture vessels, however, would include laboratory-specific culture jars, Erlenmeyer flask, Magenta bottles, or screw-capped test tubes which would cost at least PhP150 per piece. Hence, at least 85% of the cost has been reduced when using food jars. These potential alternatives can therefore find wide acceptability in developing countries that practice micropropagation.

TABLE 6 Cost comparison of the different combinations of culture vessels and sealing materials used for in vitro culture of coconut (*Cocos nucifera* L.) cv. Laguna Tall

Sealing material	Relative cost per bottle size (PhP)*		
	45 mL	60 mL	100 mL
Polypropylene sheets with rubber band	10.68	22.68	22.68
Aluminum foil with masking tape	7.32	19.32	19.32

*Based on seller prices in 2017 purchases excluding cost of *Cocos nucifera* embryo and medium

Conclusion

Culture in 3 d under Eeuwens liquid medium with sugar (sucrose or white table sugar) or without (distilled water) resulted in plumule protrusion. Hence, the addition of sucrose or table sugar may be optional for plumule initiation. Smallest glass culture vessels (45 mL) or aluminum foil as sealing material produced highest number of healthy explants. Calli were produced consistently from 45-mL vessels and sealing material with aluminum foil and masking tape produced highest percentage of white calli. Cost analysis revealed that smallest (45 mL) glass jar as culture vessel and aluminum foil with masking tape as sealing materials were cheaper alternatives at PhP 7.32 compared to using a 100-mL glass vessel with polypropylene sheet and rubber band at PhP 22.68.

Recommendation

As contamination was high, this study recommends further reduction of contamination by fumigating the laboratory once a week, wiping down the laminar flow hood with 70% ethanol after exposing it to UV for 30 min, and cleaning of high-efficiency particulate air (HEPA) filter every quarter. This study also recommends increasing the sample population and extending the research to cover tissue development up to plantlet production.

Note

This paper reports on results from the undergraduate theses of Ms. Dejan and Ms. Asumbrado, which were submitted for a degree in BS Biology at the University of the Philippines Mindanao.

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