ASIA LIFE SCIENCES 24(1): 111-125, 2015
The Asian International Journal of Life Sciences

Tissue culture technique for clonal propagation of nipa palm (*Nypa fruticans* Wurmb., Arecaceae) from embryo culture

VICTORIA C. LAPITAN[1*], KATRINA LESLIE C. NICOLAS[1] and EUFEMIO T. RASCO, JR.[1]

In the Philippines, nipa palm (*Nypa fruticans* Wurmb., Arecaceae) has been identified as an important source of biofuel. It produces high amount of sap that can be converted to bioethanol. However, making industrial alcohol from nipa is hampered by the availability of superior planting materials in a large quantity. *In vitro* clonal propagation is a promising alternative to produce such materials over a short period of time. This is the first attempt to develop a micropropagation technique for nipa using *in vitro* cultured embryos from mature fruits. Washing of explants with high amount of powdered detergent prior to sterilization under aseptic condition resulted to significant increase in decontamination percentage. Modified Euwens (MY3) basal medium enhanced higher percentage of embryo germination than MS medium. Addition of coconut water did not increase germination rate but it promoted a healthy development of shoots with normal leaves. Clonal propagation was performed on plantlets with 2-3 primordial leaves through cutting along the shoot apical meristem. Clones were generated after 3 months on MY3 medium with higher concentration of cytokinin than auxin. Through this technique, it is possible to produce 600 superior plants from one fruiting head per year at 80% survival while conventionally, 54-85 seedlings can be generated from the same number of fruit at 60-93% germination.

***Keywords*:** biofuels, *in vitro* clonal propagation, nipa palm, *Nypa fruticans*

[1]Philippine Rice Research Institute Los Baños, College 4031, Laguna, Philippines
[*]Corresponding author: *e*-mail - vc.lapitan@philrice.gov.ph

Received 03 July 2013; Accepted 19 August 2013

Printed in the Philippines

INTRODUCTION

Nipa (*Nypa fruticans* Wurmb., Arecaceae) is the most common, widely distributed and useful palm found in the mangrove forests in Southeast Asia (Tsuji et al. 2011). Similar to its relative, coconut palm, it has multiple uses; every structure can be utilized for variety of purposes. Several authors have documented different uses of nipa from young leaves, petioles, main axes, leaflet midribs, stalks, seeds, buds and petals ((Baja-Lapis et al. 2004, Burkill 1935, Fong 1984, 1992; Päiväke 1996, Robinson 1911, Whitmore 1973 and Fong 1992 as cited by Tsuji et al. 2011). In the Philippines, nipa palm has been identified as an important source of biofuel because it produces high amounts of sugar-rich sap that can be converted to alcohol upon fermentation. It can yield alcohol at least 3-4 times as that of sugar cane, the main source of ethanol in the country. Rosario (1982) reported that 1 hectare of nipa can produce as much as 26,000 liters of pure alcohol per year. By comparison, sugar cane gives only 3350-6700 liters and cassava, 3240-8640 liters. Production of biofuel from nipa is gaining scientific attention in support of the government's desire to reduce the country's dependence on imported oil, and provide a cheaper, more environment-friendly alternative to fossil fuels. Having seen this potential, development of cultivars with high sap yield will be an important objective of improving nipa for increased alcohol productivity. In addition, based on survey (Rasco 2011), there was substantial variation in sap production and sap yield related parameters between and among nipa plants in two locations in the country, Camarines Norte and Surigao del Norte, Philippines. This variation can be exploited in developing improved populations (or varieties) and hybrids that can be useful in future programs to expand the utilization of nipa for economic activities. However, nipa is believed to be highly cross pollinated and normally propagated by seeds, whose breeding cycle usually takes a lot of time. Because of this pollinating habit, nipa plants generated from seeds are diverse. Likewise, large scale cultivation of high yielding uniform nipa plants is difficult to obtain through seed propagation.

Embryo culture is a useful technique to shorten the breeding cycle and at the same time to propagate plants vegetatively. It is one of the earliest *in vitro* culture techniques applied to practical problems and has proven of greatest value to breeders (Dunwell 1986). Embryo culture is a convenient initial source for the establishment of shoot cultures because of their juvenile nature (Chaturvedi et al. 2004). Younger tissues, such as zygotic embryos are usually the preferred choice for tissue culturists as they have higher potential and are more potent to produce embryos and organs compared to more differentiated and mature tissues (Elhiti & Stasolla 2011). Rapid multiplication of oil palm cv. Tenera was successful using cultured zygotic embryos as starting material (Thuzar et al. 2011). Micropropagation of *Heliconia bihai* L. was also established using zygotic embryos excised from either immature or mature fruits as starting material (Ulisses et al. 2010). Embryo culture involves isolating and growing an immature or mature zygotic embryo under sterile conditions on an aseptic nutrient medium with the goal of obtaining a viable plant (Bridgen 1994). The integrity of the hybrid genome is retained in an embryo and therefore utilizing the cultured embryos as starting material for clonal propagation is possible.

Embryo tissue culture of nipa palm

In vitro clonal propagation provides a promising alternative for cloning large quantities of superior planting materials within a short period of time (Thorpe et al. 1991) throughout the year without seasonal constraints. Nipa clones offer the potential for higher alcohol productivity because it is possible to establish uniform trees comprised of identical clones of highly productive genotypes. Tissue culture technology is extensively used as a biological tool for clonal and mass propagation of date palm (Al-Khalifah et. al 2012, Abul-Soad & Mahdi 2010) and coconut palm (Blake & Homung 1995). In developing a reliable cloning technique for use in the breeding program for coconut, studies on vegetative propagation were undertaken using explants such as immature inflorescence (Branton & Blake 1983), tender leaf (Raju et al. 1984) and immature zygotic embryo (Karunaratne & Periyapperuma 1989). On the other hand, Hornung (1995) and Chan et al. (1998) tried plumules (embryo meristem and first leaves) and observed them to be better than that of other tissues. For date palms, explants that can be used successfully for micro propagation includes inflorescence, leaf segments, stem and root sections.

For nipa, there has been no established clonal propagation protocol yet; hence, this study was conducted to develop methods for clonal propagation from *in vitro* cultured embryos to generate uniform, genetically stable and true-to-type planting materials over a short period of time.

MATERIALS AND METHODS

Time and place of the study. The study consisted of three activities namely microbial decontamination of explants, germination of zygotic embryos and clonal propagation. All the experiments were conducted from January to December 2013 at Tissue Culture Laboratory of Philippine Rice Research Institute Los Baños, Philippines.

Plant materials. Fruiting heads of nipa collected from Calauag, Quezon, Philippines were used as sources of explants in this study. Figure 1 describes the plant material and the process of excising the embryo utilized in the study. One fruiting head could have as many as 90 seeds but only mature and viable seeds were selected and utilized in the study (Figure 1A). The mature seed was dark brown in color and the husk was fibrous (Figure 1B & 1C). The hardened endosperm of mature seeds was white when newly opened and usually turn brown with long exposure in the air (Figure 1D). At this phase, the zygotic embryo (Figure 1E) is at the heart stage of development where the apical layer that will generate shoot meristem and the lower layer that will produce root meristem were very distinct.

Microbial decontamination of explants. Mature seeds were individually detached from the fruit stem, washed with soap and water and then rinsed with running water. The seeds were dehusked to expose the endosperms. The endosperms were cut into cubes while keeping the embryos protected and intact in its position. A total of 480 endosperms were prepared, where half of it was subjected to pre-treatment wherein the explants were further washed with soap and water by soaking in water-detergent mixture with 500 g of powdered detergent for 20 min while the other half did not undergo pre-treatment prior to aseptic sterilization. The pre-treated

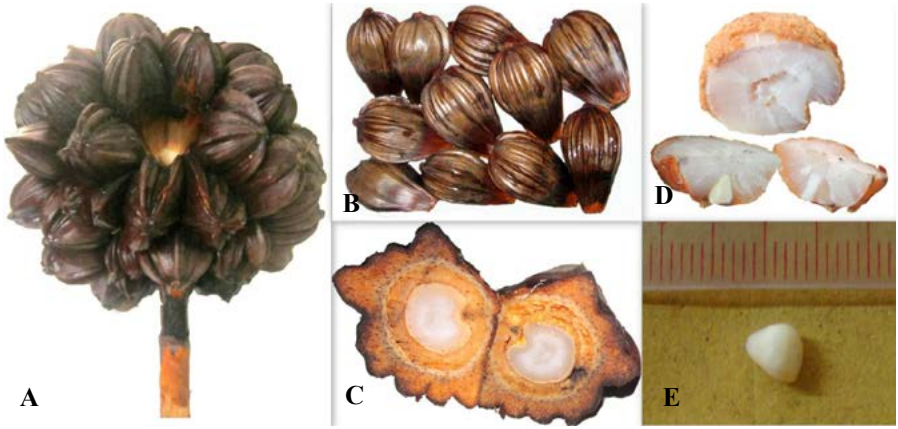

Figure 1. Planting materials utilized in the study. A. Fruiting head of nipa; B. Mature seeds detached from the fruit stem; C. Cut seeds showing the hardened endosperm; D. Cut endosperm excised from the seed; E. Zygotic embryo used as explant.

explants were then rinsed in running water until the soap suds were removed. Subsequently, the explants from both treatments i.e. with and without pre-treatments, were subjected to the next decontamination scheme where they were soaked either for 20 or 30 min in two concentrations of the active ingredient of commercial bleach i.e. 2.625 vs. 5.25% NaOCl under aseptic condition. The experiment was laid out in three-factor factorial experiment in CRD making the initial decontamination procedure as factor A, varying concentration of the active ingredient of commercial bleach as factor B and the soaking time as factor C. Prior to the dissection and excision of the embryos, the explants were further soaked in sterile distilled water thrice for 20 min per soaking. Sterile embryos were excised and inoculated in test tubes with 10 ml solidified plain Modified Euwens medium (MY3, Euwens 1978) devoid of any growth hormones but supplemented with 30g/L^{-1} sucrose. All the cultured embryos were kept at 22±2ºC under dark condition for at least four weeks. The experiment was laid out in three-factor factorial in CRD with a total of eight treatment combinations. Four replicates were made for every treatment combinations with 15 embryos per replicate. The number of sterilized embryos that survived as indicated by its swelling was recorded.

Germination of zygotic embryos. The sterilized embryos measuring 3-5 mm were inoculated onto different experimental media. Two basal media were tested, MY3 and MS (Murashige & Skoog 1962), which were supplemented either with or without 7 mg L^{-1} 2,4-D and 100 ml L^{-1} coconut water, along with 40 g L^{-1} sucrose and 2 L^{-1} activated charcoal (AC). Table 1 shows the composition of MY3 and MS media in terms of the macro- and micro-nutrients, Fe source, vitamins, and amino acids. The pH of the media was adjusted to 5.8 prior to adding the agar. All the cultures were kept at room temperature 22±2°C under dark condition and germination was observed starting from 2-4 weeks upon culture. The experiment was laid out in

Table 1. Nutrient composition of the two basal media utilized in the study.

Chemicals	Concentration (mg L^{-1}) Modified Y3(MY3)	MS
Macro-nutrients		
NH_4NO_3	-	1,650.00
KNO_3	2,020.00	1,900.00
KCl	1,492.00	-
NH_4Cl	535.00	-
$KH_2.PO_4$	-	170.00
NaH_2PO_4	312.00	-
$MgSO_4$	247.00	370.00
$CaCl_2$	294.00	440.00
Micronutrients		
$MnSO_4$	11.20	6.20
KI	8.30	22.30
$ZnSO_4$	7.20	8.60
H_3BO_3	3.10	0.83
$CoCl_2.6H_20$	0.24	0.25
$Na2MoO_4.2H_2O$	0.24	0.03
$CuSO_4.5H_2O$	0.25	0.03
Iron Source		
$FeSO_4$	27.80	27.85
Na2EDTA	37.30	37.25
Vitamins		
Myo-inositol	100.00	100.00
Thiamine-HCl	1.00	0.50
Nicotinic acid	1.00	0.10
Pyridoxin-HCl	1.00	0.50
Glycine	0.00	2.00
Amino Acids		
I-Glutamine	100.00	-
I-arginine	121.00	-
I-asparagine	88.00	-
Sucrose	40g L^{-1}	40g L^{-1}
Agar	5g L^{-1}	5g L^{-1}
AC	2g L^{-1}	2g L^{-1}

three-factor factorial in CRD, making the basal media (MY3 vs MS) as factor A; presence of 2,4-D (0 vs 7mg L^{-1}) as factor B; and presence of coconut water (0 vs 100 ml L^{-1}) as factor C.

There were eight replications per treatment combinations with 12 embryos per replicate. Data on the number of embryos that germinated as evidenced by the emergence of plumule were recorded weekly.

Clonal propagation. Germinated embryos were transferred to lighted condition (9 hr) at 22±2°C, to enhance shoot growth and development. Green plantlets with 2-3 primordial leaves, generated from embryo cultures were utilized for clonal propagation. The shoots were cut longitudinally into two sections along the shoot apical meristem and subcultured onto different growing media using MY3 as the basal salts. MY3 was identified to be the better basal medium for plant growth from the previous experiment thus it was utilized on this trial. The following combinations of the plant growth hormones; 6-benzylaminopurine (BAP), 1-naphthaleneaceticacid (NAA), and kinetin (Ki), in various concentrations along with 0.1g L^{-1} myo-inositol, 45g L^{-1} sucrose, 2.5g L^{-1} activated charcoal (AC) were supplemented on the MY3 medium:

(1) 0 BAP + 1.0 mg L^{-1} NAA + 1.0 mg L^{-1} Ki
(2) 0.5 mg L^{-1} BAP + 1.0 mg L^{-1} NAA + 1.0 mg L^{-1} Ki
(3) 0.5 mg L^{-1} BAP + 1.0 mg L^{-1} NAA + 0 Ki
(4) 1.0 mg L^{-1} BAP + 1.0 mg L^{-1} NAA + 1.0 mg L^{-1} Ki
(5) 1.0 mg L^{-1} BAP + 2.0 mg L^{-1} NAA + 1.0 mg L^{-1} Ki

All cultured clones were incubated under lighted condition (9 hr) at 22±2°C. A total of 18 clones per experimental media were cultured. The number of clones which regenerated new shoots and developed roots was recorded.

Statistical analysis. Statistical analyses of all collected data from the above three experiments such as decontamination rate, embryo germination rate, and number of shoot and root formation were performed using SAS 9.1 to determine the differences between treatment means at 5% level of significance and Microsoft Excel Statistical software.

RESULTS AND DISCUSSION

Microbial decontamination of explants. Establishing aseptic explants/tissues was a primary concern in any *in vitro* propagation technique. Explants collected from the field should as much as possible be free from contaminants such as bacteria, virus, fungi and other contaminants. Initial washing of nipa fruits with soap and water prior to dehusking did not guarantee that the explants were already free from contaminants. In this study, decontamination of zygotic embryos of nipa was carried out using powdered detergent for pre-treatment and then followed by commercial bleach (Zonrox) treatment in establishing aseptic embryo cultures for nipa.

The best possible combination of these sterilants for embryo culture was selected based on explants that showed the highest rate of survival and had the least contamination. Results showed that washing of explants with powdered detergent prior to aseptic sterilization significantly increased the decontamination percentage

of the explants to almost 120% (Table 2) as compared to those not subjected to pre-treatment. Through this process, grime, soils and other dirt accumulated during the cutting of endosperms, which could harbor contaminants such as fungi and bacteria might have been removed during the process. The procedure minimizes the possible source of contaminants present prior to sterilization under aseptic condition. The results of this experiment revealed that the pre-treatment method was effective in establishing aseptic cultures of nipa embryos since all cultured embryos were decontaminated whereas only 41.6-50.0% of the total number of embryos was disinfected in without pre-treatment.

Table 2. Decontamination rate of zygotic embryos in two NaOCl concentrations soaked in varying time, with and without pre-treatment prior to sterilization under aseptic condition.

% NaOCl	Soaking time (min)		Average decontamination rate across treatments
	20	30	
Without pre-treatment			
2.62	41.67	48.33	
5.25	43.33	50.00	45.83[b]
With pre-treatment			
2.62	100	100	
5.25	100	100	100[a]

Mean separation by LSD at 5% level

The effective concentration of NaOCl was also tested since commercial bleach (sodium hypochlorite, NaOCl) is the most frequent choice for surface sterilization because it is affordable and readily available. It is also safe to use and does not require special handling and waste-disposal precautions, therefore making it both researcher- and environment-friendly disinfectant (Maina et al. 2010). However, result showed that level of NaOCl concentration had no significant effect on decontamination rate of the embryos. Several published protocol in decontaminating palms' embryos also utilized sodium hypochlorite but in tandem with 70% ethanol. Guerra and Handro (1998) utilized 4% sodium hypochlorite and 70% ethanol in sterilizing zygotic embryos of *Euterpe edulis* (Palmae). Saenz et al. (2006) also used sodium hypochlorite in obtaining aseptic embryo culture of coconut where the plumules explants were excised. The effectiveness of commercial bleach with NaOCl as active ingredient is attributed to the fact that when the hypochlorite salts (NaOCl) were diluted with water, they were formed into hypochlorous acid (HOCl) whose concentration is correlated with bactericidal activity (Nakagarwara et al. 1998). The germicidal properties of HOCl have been well reported (Lapenna & Cuccurullo 1996, McKenna & Davies 1988).

All the disinfected embryos from all treatments survived and continued to grow into viable plants. Growth was observed on the cultured embryos as evidenced by the emergence of plumule (Figure 2) after three weeks of incubation.

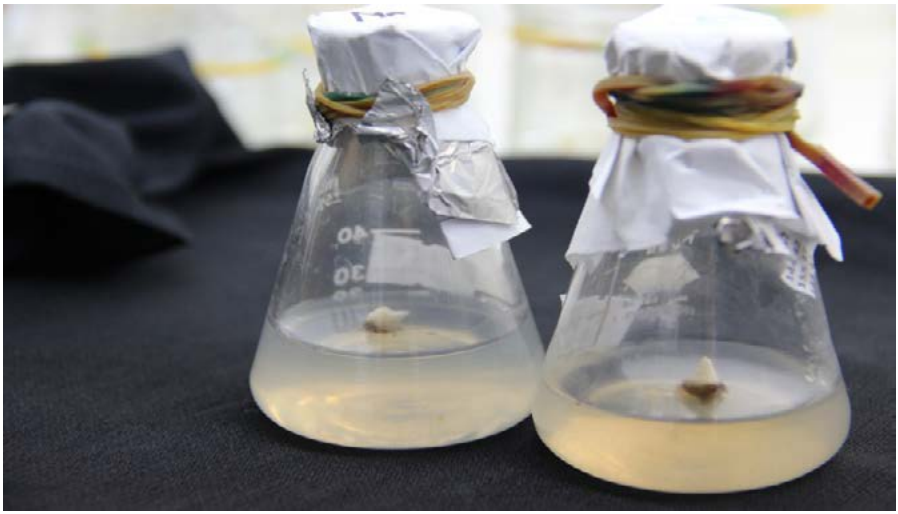

Figure 2. Emergence of plumules from cultured embryos after three weeks of incubation in the dark.

Germination of zygotic embryos. There was no established protocol for *in vitro* culture of nipa yet, therefore, the commonly used basal media such as MS and MY3, the medium tested for coconut and other palms (Saenz et al. 2006, Ashburner et al. 1995, Areza-Ubaldo et al. 2003, Sogeke 1998, Verdeil et al. 1994, Pannetier & Buffard-Morel 1982) were evaluated. Swelling and expansion of zygotic embryos became obvious two weeks after culture followed by the emergence of plumule within the following week under dark condition (Figure 3A). Once the germinating seedlings were transferred under lighted condition, they started to turn green and form new leaves. Although the observed pattern of growth and development was quite similar for oil palm (Suranthran et al. 2011) var. Tenera (Thawaro & Techato 2010), nipa had longer time to develop shoots. Likewise, unlike the oil palm which performed better under lighted condition, the growth of nipa embryos was favourable under dark condition.

Analysis of data revealed that basal media greatly affects germination of zygotic embryos. There was no significant interaction effect among the three factors being evaluated such as basal media, presence and absence of 2, 4-D (7 mg L^{-1}) and coconut water (100 ml) on the germination of the zygotic embryos. As shown in Table 3, from among 12 embryos replicated 8 times, higher mean germination percentage (89.07%) was observed on MY3 compared with MS medium (78.12%).

Figure 3. Clonal propagation of nipa. A. Germinated zygotic embryos after one month of incubation in the dark; B. Green plantlets generated from cultured embryos; C. Green plantlets cut longitudinally into two sections along the shoot apical meristem.

MY3 has higher source of amino acids as compared with MS; which might be the reason for the observed difference on the germination percentage of the zygotic embryos. The observed differences on basal media tested was significantly different from each other ($p<0.05$). The use of MY3 basal salts increased the germination of nipa embryos by almost 11%. The results showed the potential MY3 for the germination of zygotic embryos of nipa. The result, however, was not in agreement with the findings of Thawaro and Te-chato (2010) for oil palm where MS medium has been widely used as a culture medium for growth and development of zygotic embryos.

The addition of activated charcoal on culture medium was also performed to enhance growth and development of developing embryos since various authors have shown the valuable benefits of incorporating activated charcoal in tissue culture medium. Karunaratne et al. (1985) observed that a significant retardation

Table 3. Zygotic embryo germination in MS and MY3 basal media supplemented with 2, 4-D and coconut water.

2,4-D concentration (mg L^{-1})	Germination (%) [a]			
	MS		MY3	
	(-) coconut water	(+) coconut water	(-) coconut water	(+) coconut water
None	81.25	81.25	87.50	93.75
7.0	71.88	78.13	84.38	90.63
Average germination rate across basal media	78.12 b		89.07 a	

[a]Average of eight replications. Mean separation by LSD at 5% level

in growth of coconut embryos and complete cessation of haustarium development and root initiation occurred when charcoal is omitted from the medium. Assy-Bah and Engelmann (1993) claimed that germination of coconut embryos and their further development depended greatly on the presence of activated charcoal. De Guzman et al. (1971) and Rillo and Paloma (1990) have also shown the importance of activated charcoal as medium additive for the germination and development of coconut embryos. Similarly, Suranthran et al. (2011) observed that addition of activated charcoal into the growing medium enhanced the *in vitro* growth and development of oil palm zygotic embryos. It has been suggested by several authors (Constantin et al. 1977, Martineau et al. 1981, Wang & Huang 1976 as cited by Karunaratne et al. 1985) that the beneficial effect of charcoal may be attributed to its adsorption capability in removing inhibitors and excessive quantities of tissue-produced and exogenously supplied growth regulators in the culture medium.

Cytokinis and auxins are the commonly used plant growth regulators in many tissue culture activities. Of all the auxins or auxin-like plant growth regulators, 2,4-D has proven very effective in most of the embryogenic studies on several crops (Evans et al. 1981, Du et al. 2007, Karunaratne et al. 1990). In the present study, the effect of 2,4-D on the germination of zygotic embryos was tested. Although the result was not significant, the presence of 2,4-D in the medium decreased the rate of embryo germination. Based on the study of Zheng and Sun (2009), however, 2,4-D significantly suppressed the seed germination of huagaimu with only 2% germination rate after 50 d of incubation. The negative effects of 2, 4 -D on seed germination were observed in various species (William & Hoagland 1982, Sathiyamoorthy 1990, Khan & Aslam 2006).

Although the effect was not significant, the growth-promoting capability of coconut water on nipa embryos was observed when supplemented on MY3 medium only. Addition of coconut water resulted to a germination percentage of almost 94% and also promoted a healthy development of shoots with normal leaves (Table 3). Nasib et al. (2008) and Mohammad et al. (2010 as cited by Muhammed et al. 2013) also reported that coconut water effectively enhance plantlet development. Wide application of coconut water including growth supplement in plant tissue

culture/micropropagation can be justified by its unique chemical composition of sugars, vitamins, minerals, amino acids and phytohormones (Yong et al. 2009). Coconut water is also a rich source of cytokinins or cytokinin-like substances that promoted multiple shoot induction for clonal propagation of orchid (Te-chato et al. 2009) and banana (Gupta 1986).

Clonal propagation. Figure 3A-C show the clonal propagation scheme for nipa using zygotic embryos as starting materials. Green plantlets with 2-3 primordial leaves (Figure 3B) generated from previous experiment were cut longitudinally into two sections along the shoot apical meristem (Figure 3C) and cultured in MY3 basal medium with varying concentrations of hormones like auxin such as NAA and cytokinin such as BAP and Ki. The percentages of clone with shoot and root formations were not significantly affected by BAP-NAA-Ki combination in the propagation media (Table 4). However, it was observed that increasing the concentration of BAP to 1.0 mg L^{-1} in combination with the same concentration of NAA and Ki increased the number of clones forming shoots (Figure 4A & B) but no root formation was observed. Out of the total 18 cut plantlets cultured on the said medium, 27.8% formed shoots. Generally, cytokinin induces shoot formation using shoot tips or axillary explants in vitro both in monocots and dicots. Although the response of different plant species in terms of shoot formation varied with different kind and concentration of cytokinin, several reports proved that among cytokinins, BAP was the most effective for this purpose (Jafari et al. 2011, Hassan et al. 2011, Mazri 2012). For oil palm, Inpeuy et al. (2011) observed otherwise. Results of their study showed that BAP and other cytokinins such as kinetin and thidiazuron, were not effective in the induction of multiple shoots from cultured half shoot. However, incorporation of coconut water into the basal medium induced more shoots with normal leaves. Root formation (Figure 4C), was also observed in one of the experimental media. This was observed on medium with reduced concentration of BAP. However, out of the 18 clones cultured on this medium, only one was able to exhibit that response. The primary root was 5 cm long and quite vigorous. The result was quite similar with Liang (2007) who reported that low concentration of auxin and high cytokinin in the medium resulted in the induction of shoot mopho-

Table 4. Shoot and root formation of nipa clones as affected by different concentrations of BAP, NAA and Ki.

BAP (mg L^{-1})	NAA (mg L^{-1})	Ki (mg L^{-1})	% Clones with shoot formation	% Clones with root formation
0	1.0	1.0	11.1	-
0.5	1.0	1.0	11.1	5.6
0.5	1.0	0	5.6	-
1.0	1.0	1.0	27.8	-
1.0	2.0	1.0	16.7	-

*Using a total of 18 clones per treatment.

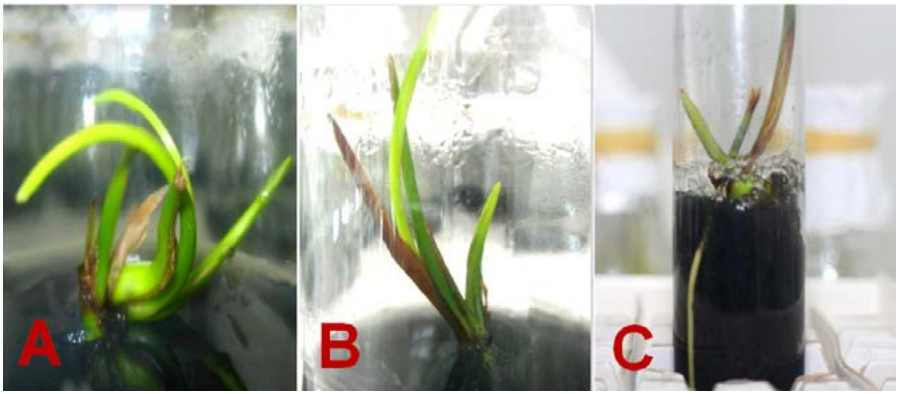

Figure 4. Shoot and root formation of nipa clones. A & B. Regeneration and growth of new leaves; C. Root formation and elongation of cultured nipa clone.

genesis while the presence of auxin alone or with the presence of very low concentration of cytokinin promoted root induction for *Phyllanthus niruri* plantlets (Euphorbiaceae). Al-Khateeb (2006) also got similar results for date palm (*Phoenix dactylifera* L.) cv. Sukry.

Results of the study indicated that it is possible to clonally propagate nipa seedlings through shoots regenerated from embryos by cutting it longitudinally along its apical meristem. Similarly for oil palm, adventitious shoot formation was observed when the cut explants or clones from zygotic embryos were cultured in a liquid MS medium supplemented with 15% coconut water, BAP and Ki (Inpeuy et al. 2011). Longitudinal cutting resulted to production of true-to-type sibling plants in a short period of time. For mass propagation of banana, the explants were likewise cut longitudinally to break the apical dominance prior to inoculation on shoot multiplication medium (Sandoval et al. 1993).

CONCLUSION

The results of this study indicate that it is possible to utilized *in vitro* cultured zygotic embryos as explant for clonal propagation of nipa through cuttings longitudinally along the apical meristem. In the preparation of explant, it is important to wash it first with high amount of detergent powder prior to sterilization under aseptic condition using NaOCl. Initiation of zygotic embryos was highly enhanced using modified Euwens (MY3) basal media. Germinated embryos should then be transferred to lighted condition after germination to enhance the growth and development of shoots. The green plantlets with 2-3 leaf primordial is suited to be an explant for clonal propagation and clones with new shoots are possible to be regenerated on MY3 basal media as long as it is supplemented with same concentration of BAP, NAA and Ki and clones with roots are also regenerated on the same basal media with reduced BAP concentration.

Further, the results of the study implied that from one seed of nipa it is possible to generate cloned seedlings through *in vitro* clonal propagation in shorter period of time. Through this newly developed technique for nipa, it is possible to

produce 600 superior plants from one fruiting head per year at 80% survival while conventionally; 54-85 seedlings can be generated from the same number of fruit at 60-93% germination. Likewise, the technique offers a system of producing uniform and genetically pure materials of nipa plants that can be utilized or planted for commercial purposes. Planting of such ensures uniform growth, easy harvesting and management, and increase yield. However, commercial micropropagation of nipa through tissue culture still needs a lot of research, exploration, and funds to further basic researches on this especially on improving the response of cloned nipa plantlets *in vitro*.

ACKNOWLEDGMENTS

The authors wish to thank Department of Agriculture-Regional Field Office V (DA-RFO V) and Philippine Rice Research Institute (PhilRice) for providing financial support in the conduct of this study and also to the Staff of the Tissue Culture Laboratory (TCL), Plant Breeding and Biotechnology Division (PBBD), PhilRice Los Baños for their tireless efforts and generous assistance in carrying out the experiments.

LITERATURE CITED

Abul-soad, A.A. and S. Mahdi Mohd. 2010. Commercial production of tissue culture date palm (*Phoenix dactylifera* L.) by inflorescence technique. Journal of Genetic Engineering and Biotechnology 8(2): 39-44.

Al-khalifah, N.S., E. Askari and A.E. Shanavaskhan. 2012. Date palm tissue culture and genetical and morphological identification of cultivars grown in Saudi Arabia. King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia, 264 p.

Areza-Ubaldo, M.B., E.P. Rillo and C.A. Cueto. 2003. Application of the improved embryo culture protocol for commercial production of makapuno seedlings. Philippine Journal of Science 132(1): 1-11.

Ashburner, G.R., M.G. Faure, D.R. Tomlinson and W.K. Thompson. 1995. A guide to zygotic embryo culture of coconut palms (*Cocos nucifera* L.). ACIAR Technical Reports No. 36.

Assy-Bah, B. and F. Engelmann. 1993. Medium-term conservation of mature embryos of coconut. Plant Cell Tissue and Organ Culture 33: 19-24.

Blake, J. and R. Homung. 1995. Somatic embryogenesis in woody plants, pp. 327-340. *In:* Jain, S., P. Gupta and R. Newton (Editors). Volume 2, Kluwer Academic Publishers, The Netherlands.

Branton, R.L. and J. Blake. 1983. Development of organized structures in callus derived from explants of *Cocos nucifera* L. Annals of Botany 52: 673-678.

Bridgen, M.P. 1994. A review of plant embryo culture. Hortscience 29(11): 1243-1246.

Chan, J.L., L. Saenz, C. Talavera, R. Hornung, M. Robert and C. Oropesa. 1998. Regeneration of coconut (*Cocos nucifera* L.) from plumule explants through somatic embryogenesis. Plant Cell Reproduction 17: 515-521.

Chaturvedi, R., M.K. Razdan and S.S. Bhojwani. 2004. *In vitro* morphogenesis in zygotic embryo cultured of neem *(Azadirachta indica* A. Juss). Plant Cell Reproduction 22(11): 801-809. Doi: 10.1007/s00299-004-0768-0[Pubmed][Cross Ref].

De Guzman, E.V., A.G. Del Rosario and E.C. Eusebio. 1971. The growth and development of coconut 'Makapuno' embryo *in vitro*. Resumption of growth in high sugar media. Philippine Agriculturist 53: 566-579.

Rosario, E.J. Del. 1982. Fuel alcohol from agricutural crops and residues. Regional Workshop on the Technology of Fuel Alcohol Production, University of the Philippines Los Banos, College, Laguna. Philippines. (unpublished)

Dunwell, J.M. 1986. Pollen, ovule and embryo culture as tools in plant breeding, pp. 375-404. *In:* Withers, L.A. and P.G. Alderson (Editors). *Plant Tissue Culture and its Agricultural Applications*, Butterworths, London, UK.

Eeuwens, C.J. 1978. Effects of organic nutrients and hormones on growth and development of tissue explants from coconut (*Cocos nucifera*) and date (*Phoenix dactylifera*) palms cultured *in vitro*. Plant Physiology 42: 173-178.

Elhiti, M. and C. Stasolla. 2011. The use of zygotic embryos as explants for *in vitro* propagation: An overview. *In:* Trevor, A., E. Thorpe and C. Yeung. (Editors). *Plant Embryo Culture: Methods and Protocols*. Volume no. 710, Print ISBN:978-1-61737-987-1.

Guerra, M.P. and W. Handro.1998. Somatic embryogenesis and plant regeneratopn in different organs of *Euterpe edulis* Mart. (*Palmae*): Control and structural features. Journal of Plant Research 111: 65-71.

Hornung, R. 1995. Micropropagation of *Cocos nucifera* L. from plumular tissues excised from mature zygotic embryos. Plantations, Recherche, Development 212: 38-41.

Karunaratne, S., C. Kurukulaarachchi and C. Gamage. 1985. A report on the culture of embryos of dwarf coconut, *Cocos nucifera* L. var. *nana in vitro*. Cocos 3: 01-08.

Karunaratner, S., and K. Periyapperuma. 1989. Culture of immature embryos of coconut, *Cocos nucifera* L.: Callus proliferation and somatic embryogenesis. Plant Science 62:247-253.

Mohammad, S., and M. Ali. 2010. Effect of coconut water on callus growth of *Cyamopsis tetragonolobus*. Pharmacia 1(1): 25-27.

Muhammed, N., R. Nyamota, S. Hashim and J. Malinga. 2013. Zygotic embryo *in vitro* culture of *Cocos nucifera* L. (cv. East Afrtican Tall variety) in the coastal lowlands of Kenya. African Journal of Biotechnology 12(22): 3435-3440.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiology 15: 473-497.

Nasser, S. and A.E. Shanavaskhan. 2012. Micropropagation of date palms. Asia-Pacific Consortium on Agricultural Biotechnology (APCoAB) and Association of Agricultural Research Institutions in the Near East and North Africa (AARINENA), 54 p.

Pannetier, C. and J. Buffard-Morel. 1982. Production of somatic embryos from leaf tissues of coconut, *Cocos nucifera* L., pp. 755-756. *In:* Proccedings of Fifth International Congress on Plant Tissue and Cell Culture, 1982.

Raju, C.R., K.P. Prakash, M. Chandramohan and R.D. Iyer. 1984. Coconut plantlets from leaf tissue cultures. Plantation Crops 12: 5-81.

Rillo, E.P. and B.F. Paloma. 1990. Comparison of three media formulations for *in vitro* culture of coconut embryos. Oléagineux 45: 319-323.

Sáenz, L., A. Azpeitia, B. Chuc-armendariz, J.L. Chan, J.L. Verdeil, V. Hocher and C. Oropeza. 2006. Morphological and histological changes during somatic embryo formation from coconut plumule explants. *In Vitro* Cellular Development Biology-Plant 42: 19-25.

Sogeke, A.K. 1998. Stages in the vegetative 'propagation of oil palm, *Elaeis guineensis* Jacq. through tissue culture. Journal of Oil Palm Research 10(2): 1-9.

Thorpe, T.A., I.S. Harry and P.P. Kumar. 1991. Application of micropropagation to forestry, pp 331-336. *In:* Debergh, P.C. and R.H. Zimmerman (Editors). *Micropropagation*, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Thuzar, M., A. Vanavichit, S.Tragoonrung and C. Jantasuriyarat. 2011. Efficient and rapid plant regeneration of oil palm zygotic embryos cv. 'Tenera' through somatic embryogenesis. Acta Physiologi Plantae 33: 123-128.

Tsuji, K., N.F. Ghazalli Mohd, Z. Ariffin, S. Nordin, M. Khaidizar, E. Dulloo and L. Sebastian. 2011. Biological and ethnobotanical characteristics of nipa palm (*Nypa fruticans* Wurmb.): A review. Sains Malaysiana 40(12): 1407-1412.

Verdeil, J.L., C. Huet, F. Grosdemange and J. Buffard-Morel. 1994. Plant regeneration from cultured immature inflorescences of coconut (*Cocos nucifera* L.): Evidence for somatic embryogenesis. Plant Cell Reports 13: 218-221.

Yong, J.W.H., L. Ge, Y.F. Ng and S.N. Tan. 2009. The chemical composition and biological properties of coconut (*Cocos nucifera* L.) water. Molecules 14: 5144-5164.

ASIA LIFE SCIENCES
EST. 1992
Beyond Excellence©

81 Governor F.T. San Luis Avenue, Masaya, Bay 4033
Laguna, Philippines
Celfone nos. (063) (049) 0915-360-4660; 0921-374-9752; 0916-526-0164
e-mails: asialifesciences@yahoo.com
wsmgruezo@gmail.com
http://journals.uplb.edu.ph/index.php/ALS

The papers published in ***Asia Life Sciences*** are indexed in the Biological Abstracts, CAB Abstracts, CAB Global Health, Zoological Record, SciSearch®/Science Citation Index Expanded, Journal Citation Reports/Science Edition, BIOSIS Previews, ISI Web of Science®, ISI Web of Knowledge® and are covered by the Thomson Reuters-Institute for Scientific Information (ISI), USA and CABI, Wallingford, Oxon, UK.

Asia Life Sciences has an Impact Factor of **0.259**.

Asia Life Sciences is a recipient of the Journal Accreditation Award of the Commission on Higher Education (CHED), Republic of the Philippines (2010-2015).

Printed on acid-free papers

Beyond Excellence©

81 Governor F.T. San Luis Avenue, Masaya, Bay 4033
Laguna, Philippines
Celfone nos. (063) (049) 0915-360-4660; 0921-374-9752; 0916-526-0164
e-mails: asialifesciences@yahoo.com
wsmgruezo@gmail.com
http://journals.uplb.edu.ph/index.php/ALS

The papers published in ***Asia Life Sciences*** are indexed in the Biological Abstracts, CAB Abstracts, CAB Global Health, Zoological Record, SciSearch®/Science Citation Index Expanded, Journal Citation Reports/Science Edition, BIOSIS Previews, ISI Web of Science®, ISI Web of Knowledge® and are covered by the Thomson Reuters-Institute for Scientific Information (ISI), USA and CABI, Wallingford, Oxon, UK.

Asia Life Sciences has an Impact Factor of **0.259**.

Asia Life Sciences is a recipient of the Journal Accreditation Award of the Commission on Higher Education (CHED), Republic of the Philippines (2010-2015).

Printed on acid-free papers