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Plant Regeneration Through Direct Shoot Formation From Sago Palm (*Metroxylon sagu* Rottb.) Leaf Explants

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ABSTRACT

Sago palm (*Metroxylon sagu*) is an emerging important food plant for marginal lands. The palm reproduces in the wild slowly by means of suckers. Thus, *in vitro* culture methods must be employed to aid its multiplication. In this study, varying concentrations of auxin and cytokinin were evaluated to establish appropriate media for shoot regeneration of sago palm (*Metroxylon sagu* Rottb.) leaf explants. Shoots that regenerated from modified Murashige and Skoog (MS) medium containing 10 mg L^{-1} 2,4 D and 0.2 mg L^{-1} kinetin with 0.3% activated charcoal, 3% sucrose and 2 g L^{-1} gelrite at pH 5.7 showed the greatest weight increases. However, shoot explants inoculated in media adjusted to pH 6.8 showed faster morphological changes.

Key words: *Metroxylon sagu*, tissue culture, direct shoot regeneration, plant growth regulators

INTRODUCTION

Sago palm can be utilized as food and feed as well as non-food uses such as biodegradable plastics. For income generating activities, sago palm is useful in producing roofing materials, handicrafts, sweets, bread, etc. at small-holder farmer level (Quat Ng, 2007). Thus, the sago palm may contribute to poverty alleviation, promote household food security and can revitalize rural economies when used optimally.

Conventional practices for sago palm propagation are through seedlings and suckers. Propagation with seedlings is not commonly used owing to the seed's very low germination rate. Propagation through suckers is more common but rooting takes several months. Also, the rate of rooting may be less than 50%. Sago palm takes more than ten years to flower (Hisajima, 1996). Aside from the conventional seed and sucker propagation, innovative techniques such as tissue culture by inducing callus and regeneration of plantlets is an alternative means of propagation.

Chemicals having the same physiological activities to natural plant growth substances, termed as Plant Growth Regulators (PGRs), can be produced synthetically or through fermentation (Sathyanarayana and Varghese, 2007). Since different plant species require different kinds and levels of PGRs in inducing callus and regenerating plantlets, metabolite production and growth, it is important to evaluate the most effective combination of the most appropriate PGRs in media preparation to obtain optimum growth of sago palm in tissue culture. In culture media, the requirement for growth regulators such as auxins and cytokinins varies considerably from one

tissue to another. The combination of these two plant growth regulators is important in the culture system with respect to plant morphogenesis. The ratio of auxin to cytokinin needs to be higher for embryogenesis, root initiation and callus initiation. Its reverse would lead to shoot proliferation (Sathyanarayana and Varghese, 2007).

The success of culturing plant tissues is largely dependent on the appropriate composition of its medium (Sathyanarayana and Varghese, 2007). Different types of culture media are now available with their standardized elemental compositions; the one selected will depend largely on the plant species and the tissue, cell or organ selected for culture. Multiple shoot formation from nodes of *Portulaca oleracea* was observed within two weeks on modified MS medium with 0.5 mg L^{-1} kinetin (Sharma *et al*., 2011). However, multiple shoot formation from nodal explants of *Phyllanthus niruri* (Euphorbiaceae) was induced when kinetin (0.5 and 1.0 mg L^{-1}) was used in MS medium in combination with BA (Liang and Keng, 2006).

In sugarcane, organogenic plant regeneration directly as well as via callus induction from shoot tip, were achieved. A combination of BAP and kinetin (at 1.5 mg L^{-1} each) was found to be optimum for shoot multiplication (Dash *et al*., 2011). Roy and Kabir (2007) were also successful in regenerating shoots from shoot tip explants of sugarcane.

Azad *et al*. (2004) reported successful induction of shoot buds on MS basal media supplemented with various concentrations of BAP in combination with either NAA or IBA. The highest length of shoots was obtained with leaf explants. Shoots have been shown to form directly from shoot tip explants.

Studies in other plant species reported successful direct shoot formation from shoot explants in various auxin and cytokinin combinations. Shoot proliferation was best in papaya in MS medium containing BAP 1.0 mg L^{-1} +kinetin 0.5 mg L^{-1} and BAP 1.0 mg L^{-1}+NAA 0.5 mg L^{-1} (Kabir *et al*., 2007). In *Solanum sessiliflorum*, The combination of 0.01 mg L^{-1} IAA and 5.0 mg L^{-1} TDZ induced the most intense axillary shoot proliferation in the variety Santa Luzia (Boufleuher *et al*., 2008).

Results obtained from the *in vitro* propagation of white mulberry (*Morus alba* L.) showed that the cytokinin BA was better than kinetin with respect to axillary shoot formation (Habib *et al*., 2003). In date palms, low hormone concentrations promoted the formation of buds and shoots and shoot height was found severely affected by high hormone concentration (Al-Khateeb, 2006).

The conditions of culture medium in plant tissue culture are also very important for these would dictate the culture's developmental pathway. These includes the pH of the medium where the capacity of cell cultures to utilize NH_4^+ as sole nitrogen source is dependent on maintaining it above 5.0 (Dougall and Verma, 1978).

Through *in vitro* techniques, sago palm in the Philippines can reach its greatest potential for crop establishment on a plantation scale. Vegetative multiplication through tissue culture is more rapid than other methods and only very small pieces of explants are needed to propagate embryos (George, 1993). Although, *in vitro* culture is now more advanced and there is already an established culture media preparation for tissue culture of most plants (Sathyanarayana and Varghese, 2007), these are not always reproducible, thus, the need to evaluate the optimum media combinations to produce a high degree of embryogenesis in sago palm. This may lead to the shortening of the long breeding cycle and the development of callus culture of sago palm for its rapid multiplication (Sathyanarayana and Varghese, 2007).

Riyadi *et al*. (2005) reported that the best medium for inducing somatic embryos from embryogenic callus of sago palm was the modified MS medium supplemented with 30 g L^{-1} sucrose, 1 g L^{-1} activated charcoal, 0.1 mg L^{-1} kinetin and 5 mg L^{-1} 2,4-D. Subculturing these somatic

embryos to a half-strength MS medium, without regulators, induced them to develop into normal plantlets (Riyadi *et al*., 2005). Thus, this study evaluated the effects of pH and different levels of plant growth regulators, 2,4-dichlorophenoxyactic acid (2,4-D) and N-(2-furfurylamino)-1-purine-6-amine (kinetin) on the growth of sago palm explants.

MATERIALS AND METHODS

Collection of plant material and surface sterilization of explants: Surface sterilization procedure were based on the methods of Novero *et al*. (2010). Sago palm suckers with a base diameter of about 5-7 cm were obtained from wild sago palm stands in Sta. Cruz, Davao del Sur and Agusan del Sur, Philippines. The suckers were trimmed and washed with soapy water for 1 h before treating with fungicide (Dithane M-45; 1% v/v) for 30 min. A three-step surface sterilization protocol was followed. The first step was accomplished by dipping the trimmed suckers into 15% bleach solution (with 2-3 drops Tween 20) for 1 h with constant agitation in a rotary shaker. Suckers were rinsed three times in sterile distilled water then leaf sheaths were removed, leaving one sheath to protect the shoot meristem. The second step involved dipping the trimmed suckers into 10% bleach solution (plus 2-3 drops Tween 20) for 30 min with constant agitation, rinsing thrice in sterile distilled water. The last sheath was removed aseptically under the laminar flow hood. Finally, shoot apical tissues were submerged in 10% bleach solution (with 2-3 drops Tween 20) for 5 min and then rinsed twice with sterile distilled water. Afterwards, they were immersed in 1.3 g L^{-1} citric acid solution for 10 min to prevent browning. This was followed by a dip in 75% ethanol for 5 sec, then rinsed thrice in sterile distilled water. The shoot apical tissue was cut into 5-6 sections of 10-15 mm each.

Initiation of growth on leaf explants: Leaf explants were inoculated on modified MS media with 3% sucrose, 0.01% inositol, 0.002% thiamine hydrochloride, 3% sucrose and 0.8 g L^{-1} Gelrite as gelling agent. The effects of different combinations of auxin and cytokinin were studied. The trial involved the following factors: (1) levels of auxin (mg L^{-1}): 2,4-D at 0, 5, 10 and 15; (2) levels of cytokinin (mg L^{-1}): kinetin at 0, 0.1 and 0.2; and (3) levels of pH at 5.7 and 6.8. Media not supplemented with any of the PGRs served as the controls. The pH was adjusted to 5.8 before the media were autoclaved at 15 psi at 121°C for 15 min. Cultures were kept in the culture room at 25-27°C illuminated at about 40 μmol m^{-2} sec^{-1} at a 12 h photoperiod after a brief dark period. Cultures were checked regularly for signs of growth. Two pH levels were used, pH 5.7 which was used in previously reported literatures on sago palm tissue culture and pH 6.8 which was the average soil pH in the sago palm's natural habitat.

The inoculated culture bottles were incubated in the dark at temperature of 25±2°C for 3-4 weeks. After 3-4 weeks on the induction medium, all viable explants were transferred to fresh MMS media and incubation under cool-white fluorescent lamps which provided approximately 30 μmol m^{-2} sec^{-1} for a 14 h photoperiod.

Collection of data and statistical analysis: The response of sago palm explants to the different combinations of 2,4-D and kinetin treatment were recorded three to four weeks after inoculation. Explant survival was determined as green viable tissues free of visible contamination and phenolic injury suitable for continued culturing. Explants were qualitatively measured using a devised rating scale, weekly starting from the first week after subculture. The frequencies of shoot growth

initiated on different combinations of 2,4-D and kinetin on two pH levels (5.7 and 6.8) were determined at the end of the experiments. Explants were quantitatively measured by weighing the fresh explant before each subculture.

A multiple comparison procedure, Duncan's multiple range test, was employed in the tissue culture experiments to determine the differences among treatment means at $p = 0.05$ (Riyadi *et al.*, 2005). There were 24 treatment combinations with three replicates each represented by 72 culture bottles (250 mL capacity) containing one explant per bottle. The data collected were assessed via the Wald statistics to test for the significance (5% level) of the effects of treatments.

RESULTS AND DISCUSSION

Table 1 shows the general response of shoot explants in each treatment after one subculture. Explants of Treatment 1 (no PGR) and Treatment 2 (0 mg L^{-1} 2,4-D and 0.1 mg L^{-1} kinetin) were whitish with no visible growth due to the absence of auxins in the media. Under Treatment 3 (0 mg L^{-1} 2,4 D and 0.2 mg L^{-1} kinetin), explants showed growth protrusions even in the absence of auxin. Treatment 4 showed no visible growth due to low concentration of auxin (5 mg L^{-1}) and absence of cytokinin. Most of the explants in Treatments 6 to 12 (contained both auxin and kinetin) showed enlargement or growth protrusions, corresponding to shoot regeneration.

These preliminary results showed that a very high concentration of cytokinin at 0.3 mg L^{-1} had a necrotic effect on the explant. More greenish explants were observed in treatments with a medium at pH 6.8 after light exposure following a brief dark period. The plant growth regulators were added to hasten the growth of sago palm shoot explants *in vitro*. Auxins are responsible for stimulating cell elongation in young developing shoots. When cytokinins are added alone to the culture medium, they do not have an effect on plant growth. Thus, in Treatment 1 (no PGR), shoot explants were whitish and showed no visible growth. However, in Treatment 3 (0 mg L^{-1} 2,4 D and 0.2 mg L^{-1} kinetin) which did not contain any auxin, still showed growth protrusions which may be due to the presence of endogenous auxins interacting with the added high concentration of cytokinin at 0.2 mg L^{-1} and by the nutrients supplemented by the media.

After completing a five-six week culture period, 67% of the treatment combinations initiated shoot regeneration from the explants. Table 2 shows the percentage of shoot formation of sago palm

Table 1: Response of sago palm explants to different concentrations of 2,4-D and kinetin in full-strength MS medium with pH of 5.7 or 6.8, four weeks after inoculation

			Visual observation (color)	
Treatment	2,4-D (mg L^{-1})	Kinetin (mg L^{-1})	pH 5.7	pH 6.8
1	0	0.0	Whitish with no visible growth	Whitish with no visible growth
2	0	0.1	Whitish with no visible growth	Whitish with no visible growth
3	0	0.2	Whitish with growth protrusion	Greenish with growth protrusion
4	5	0.0	Whitish with no visible growth	Slightly greenish with no visible growth
5	5	0.1	Whitish with growth protrusion	Greenish with growth protrusion
6	5	0.2	No data: Cultures are either dead or contaminated	Whitish with growth protrusion
7	10	0.0	Greenish with growth protrusion	Slightly greenish with growth protrusion
8	10	0.1	Whitish with growth protrusion	Slightly greenish with growth protrusion
9	10	0.2	Whitish with growth protrusion	Whitish with growth protrusion
10	15	0.0	Greenish with growth protrusion	Greenish with growth protrusion
11	15	0.1	Whitish with growth protrusion	Greenish with growth protrusion
12	15	0.2	Whitish with no visible growth	Whitish with growth protrusion

Table 2: Percentage (%) of shoot formation of sago palm explants as affected by the different levels of 2,4-D and kinetin in full-strength MS medium with a pH of 5.7 and 6.8. after 5-6 weeks

				pH 5.7		pH 6.8	
Treatment	2,4-D (mg L^{-1})	Kinetin (mg L^{-1})	Replicates	No. of shoots	Shoot formation (%)	No. of shoots	Shoot formation (%)
T1	0	0.0	11	0	0	0	0
T2	0	0.1	11	0	0	0	0
T3	0	0.2	11	4	36	4	36
T4	5	0.0	11	0	0	0	0
T5	5	0.1	11	3	27	3	27
T6	5	0.2	11	0	0	2	18
T7	10	0.0	11	3	27	2	18
T8	10	0.1	11	2	18	3	27
T9	10	0.2	11	4	36	3	27
T10	15	0.0	11	3	27	3	27
T11	15	0.1	11	3	27	3	27
T12	15	0.2	11	0	0	2	18

Table 3: Marginal mean weight (g) increase of sago palm explants as affected by the different levels of 2,4-D and kinetin in full-strength MS medium with a pH of 5.7 and 6.8. after 3-4 weeks of incubation

Plant growth regulator type and concentration		Mean weight increase of explants	
2,4-D (mg L^{-1})	Kinetin (mg L^{-1})	pH 5.7	pH 6.8
0	0.0	0.120	0.076
	0.1	0.170	0.207
	0.2	0.145	0.213
5	0.0	0.150	0.203
	0.1	0.167	0.300
	0.2	*	0.100
10	0.0	0.310	0.213
	0.1	0.200	0.483
	0.2	0.490	0.200
15	0.0	0.080	0.153
	0.1	0.253	0.223
	0.2	0.170	0.185

*No data: Cultures were either dead or contaminated

shoot explants as affected by the different levels of 2,4-D and kinetin in full-strength MS medium with a pH of 5.7 and 6.8. In the absence of both PGRs in Treatment 1 (0 mg L^{-1} 2,4 D and 0 mg L^{-1} kinetin), there was no visible shoot formation from the explants. Treatment 3 (0 mg L^{-1} 2,4 D and 0.2 mg L^{-1} kinetin) at both pH 5.7 and 6.8 levels had the highest frequency (36%) of shoot formation aside from Treatment 9 (10 mg L^{-1} 2,4 D and 0.2 mg L^{-1} kinetin) at 5.7 pH.

Quantitative assessment of explant growth: Shoot regeneration was mostly achieved four weeks after inoculation. Along with this growth and development, recording increases in weight was necessary to quantitatively analyze which treatment combination was more effective in initiating growth in sago palm shoot explants. Figure 1 shows the shoot formation on explants grown in full strength MS medium+25 mg L^{-1} 2,4-D+1 mg L^{-1} kinetin at various stages: (a) one, (b) two, (c) three, (d) four and (e) five months of inoculation. Table 3 shows the mean weight increase obtained from the explants in response to treatment combinations. 2,4-D at 10 mg L^{-1}

Fig. 1(a-e): Explant inoculated in full strength MS medium+25 mg L^{-1} 2, 4-D+1 mg L^{-1} kinetin after (a) 1, (b) 2, (c) 3, (d) 4 and (e) 5 months of inoculation

Table 4: Tests of model effects of 2,4-D, kinetin and pH of the media on the increase in weight of the explants

	Type III		
Model	Wald chi-square	df	Sig.
Intercept	236.121	1	0.000
2,4-D	32.052	3	0.000
Kinetin	9.725	2	0.008
2,4-D×Kinetin	2.488	6	0.870^{NS}
pH	0.393	1	0.531^{NS}
2,4-D×pH	1.644	3	0.650^{NS}
Kinetin×pH	7.015	2	0.030
2,4-D×kinetin×pH	24.193	5	0.000

^{NS}Not significant, Dependent Variable: Weight increase of explants, df: Degree of freedom

combined with 0.2 mg L^{-1} kinetin at pH 5.7 promoted the highest mean weight increase at 0.490 g after four weeks of inoculation. This was followed by 10 mg L^{-1} combined with 0.1 mg L^{-1} kinetin with pH 6.8 at 0.483 g. Statistical analysis using Wald statistics to test for the significance of the effects of treatments are shown in Table 4.

When cytokinin (kinetin) and auxin (2,4-D) were each considered as single-factor, they had a significant effect on the increase of the weight of explants (Table 4). The pH of the medium at 5.7 and 6.8 when considered as single-factor, did not have a significant effect on the increase in weight of the explants. When cytokinin and auxin were considered as single-factor, they had no significant effect on the explants' weight increase. The interaction of pH with 2,4-D had no significant effect on the weight increase of explants in contrary to the pH and kinetin interaction. But when cytokinin, auxin and the pH of the medium were combined as single-factor, they had a significant effect on the weight increase of the explants.

CONCLUSION

Sago palm shoot explants responded well to modified Murashige and Skoog's medium containing 10 mg L^{-1} 2,4 D and 0.2 mg L^{-1} kinetin with 0.3% activated charcoal, 3% sucrose and 2 g L^{-1} gelrite at pH 5.7 showing the greatest weight increases. The pH of the medium had an effect on the PGR activity. Analysis of variance showed significant differences between different treatments on their effect on the weight increase of explants. Interaction of pH with other media components appears to have dictated the effectiveness of media on growth initiation. This result emphasizes the importance of optimizing pH conditions when establishing a new tissue culture protocol. Like other palm plants, sago palm proves to be recalcitrant. Thus, long-term goals and efforts toward enhancing sago palm's response to *in vitro* manipulations must be sustained.

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