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The influence of osmotic concentration of media on the growth of Sago Palm (*Metroxylon sagu* Rottb.) *in vitro*

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Abstract

This study investigated the role of osmotic potential of media on the early growth stages of sago palm *in vitro*. There were significant effects on the growth rate of explants after four weeks of culture under different sucrose and D-sorbitol combinations. A modified Murashige and Skoog (MS) medium supplied with 22.5 g sucrose and 7.5 g sorbitol which created a low osmotic potential environment (-0.457 MPa) had significant effects on the increase in weight of explants. This osmotic potential was found optimal for the initial growth of sago palm explants *in vitro*. In contrast, explants cultured in intermediate osmotic potential media (-0.380 MPa) obtained little increase in weight. Results suggested that owing to the sago palm's ecology where it is highly adapted to flooding even by saline water, explants likewise prefer a low osmotic potential in *in vitro* culture.

Keywords: sago palm, direct shoot formation, osmotic potential

Introduction

Sago palm (*Metroxylon sagu* Rottb; Arecaceae) grows in hot and humid tropical swamplands. It is mainly found in Papua New Guinea, Indonesia and Malaysia where it is an important crop planted to about 2.4 million hectares (Rauwerdink, 1986). It is also found in other Southeast Asian countries such as Vietnam, Thailand and the Philippines. Sago palm is a high starch-yielding crop; growing and thriving abundantly even in waterlogged acidic soil where no other agricultural crop can survive. It requires a minimum amount of fertilizer and pesticide or in most cases not necessary. Since its proliferation is by means of suckers, replanting is not necessary after its mother palm's harvest (Flores, 2007). Sago palm can be utilized as food and feed and for non-food uses such as source of material for shelter and handicrafts.Conventional practices for sago palm propagation are through seedlings and suckers. Propagation of seedlings is not commonly used owing to sago palm's very low seed germination rate. Propagation through suckers is more common, but it would take several months to root and 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, techniques such as tissue culture by inducing callus and regeneration of plantlets is an alternative means of propagation. *In vitro* culture studies on sago palm have been reported since the late 1980s. The studies involved the use of embryos and young leaf tissues as explants. However, there are very limited literatures available on sago palm micropropagation. To date, only two full papers are available (Alang and Krishnapillay, 1987 and Riyadi *et al.* 2005). The establishment of a reliable protocol remains a bottleneck in sago palm breeding. In the Philippines, there is little research devoted to the improvement of this promising crop. Riyadi *et al.* (2005) and Alang and Krishnapillay (1987) published protocols for the establishment and maturation of somatic embryos in sago palm (*Metroxylon sagu* Rottb.). Alang and Krishnapillay (1987) found that Murashige and Skoog (MS) medium supplemented with 570 µmol L^{-1} of 2,4-D in combination with 0.3% (w/v) activated charcoal led to somatic embryo formation after five months in culture. Riyadi *et al.* (2005) were able to initiate embryogenic callus on MS medium containing 22.6 µmol L^{-1} 2,4-D and 1 g L^{-1} activated charcoal. When callus cultures were transferred to medium containing 4.6 µmol L^{-1} kinetin, 3.7nmol L^{-1} abscisic acid and 0.288 µmol L^{-1} GA3, somatic embryos were formed within four weeks. This study aimed to initiate an *in vitro* propagation system for sago palm by taking into consideration important growth factors such as media, osmotic concentration and pH. In future, cloned plantlets can be used to increase the plant stands of sago palm in difficult agricultural areas.

Materials and Methods

Collection of plant material and surface sterilization of explants

Sago 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. Sago 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

Table 1. Growth of sago palm explants cultured on different concentrations of 2,4-D and kinetin after 4 wk of culture.

Treatment	MS Strength	2,4-D (mg/L)	Kinetin (mg/L)	Score/ Replicate 1 2 3 4 5 6 7 8 9 10	Mean	% Survivability After 4 Weeks	% Shoot Formation
1	Half	0	0	1 0 0 1 0 0 1 1 0 1	0.50	50	0
2			0.10	1 0 0 1 0 0 1 1 1 1	0.60	60	0
3			1	0 1 0 1 0 0 0 0 3 2	0.70	70	0
4		7	0	0 1 0 1 1 1 1 0 1 2	0.80	70	0
5			0.10	1 **5** 1 **5** 0 0 1 1 1 1	1.60	80	40
6			1	1 1 0 0 1 4 4 1 1 1	1.40	80	20
7		10	0	0 1 0 1 2 0 1 1 0 1	0.70	70	0
8			0.10	0 0 1 1 1 1 1 1 1 0	0.70	70	0
9			1	1 0 4 0 2 2 2 0 3 0	1.30	60	30
10	Full Strength	0	0	1 1 1 1 1 1 1 1 0 1	0.90	90	0
11			0.10	0 1 1 0 0 1 0 1 0 1	0.50	60	0
12			1	1 1 1 1 0 1 0 0 1 1	0.70	70	0
13		7	0	1 1 1 1 1 0 1 0 1 1	0.80	80	0
14			0.10	1 0 0 1 0 1 1 1 0 1	0.60	60	10
15			1	1 1 0 1 0 1 1 1 0 0	0.60	60	0
16		10	0	1 1 1 0 0 1 1 0 0 0	0.50	50	0
17			0.10	0 1 1 1 1 1 0 2 1 1	0.90	80	0
18			1	1 0 1 1 0 0 0 0 0 1	0.40	40	0

Rating scale: (0) dead/contaminated (1) partly brown but not dead; no visible growth (2) cream-colored; no callus formed (3) cream- colored with callus < 2 cm diameter or 25-50% green with no growth protrusion (4) cream-colored with callus > 2-5 cm diameter or 50-100% green with growth protrusion of 0-5 cm long (5) cream-colored with callus >5 cm diameter or deep green with growth protrusion of >5cm long.

Table 2. Survivability of cultured sago palm leaf explants in fifteen osmotic treatments. (+) surviving explants (-) dead explants

Treatment	Sucrose, g L^{-1}	Sorbitol, g L^{-1}	Replicates 1	2	3	4	5	Total	Percentage
1	10	0	+	+	+	-	-	3	60
2	7.5	2.5	+	-	-	-	-	1	20
3	5	5	+	+	+	+	-	4	80
4	2.5	7.5	+	+	+	+	-	4	80
5	0	10	-	-	-	-	-	0	0
6	20	0	-	-	-	-	-	0	0
7	15	5	+	+	+	-	-	3	60
8	10	10	+	+	+	+	+	5	100
9	5	15	+	+	+	-	-	3	60
10	0	20	+	+	+	-	-	3	60
11	30	0	+	+	+	+	+	5	100
12	22.5	7.5	+	+	+	+	-	4	80
13	15	15	+	+	-	-	-	2	40
14	7.5	22.5	+	+	+	-	-	3	60
15	0	30	+	-	-	-	-	1	20

drops Tween 20) for 5 min and then rinsed twice with sterile distilled water. Afterwards, they were immersed in 1.3g L^{-1} citric acid solution for 10 min to prevent browning. This was followed by a dip in 75% ethanol for 5 s, 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

Sago palm 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. Two plant growth regulators were used: 2,4-Dichlor-ophenoxyacetic acid (2,4-D, an auxin, at 0, 7 and 10 mg L^{-1}) in combination with kinetin (a cytokinin, at 0, 0.1 and 1 mg L^{-1}). The pH was adjusted to 5.8 or 6.8 as required, before the media were autoclaved at 15 psi at 121°C for 15 minutes. Cultures were kept in the culture room at 25-27 ^{o}C illuminated at about 40 µmol m^{-2} sec^{-1} at a 12h 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.

Effect of osmotic concentration of culture media on explant growth

Modified MS medium containing 4.6 µmol L^{-1} kinetin, 3.7 nmol L^{-1} abscisic acid and 0.288 µmol L^{-1} GA3, where either sucrose and/or D-sorbitol were used as the carbohydrate (and by default, osmoregulator) was prepared. Sucrose was added to culture media at 29.2 mmol L^{-1}, 58.4 mmol L^{-1} or 87.6 mmol L^{-1}, which corresponded to 10, 20, 30 g L^{-1}. To test the effects of sucrose concentration, sucrose was substituted for D-sorbitol at four different concentrations. Three treatments contained only sorbitol at concentrations of 54.8 mmol L^{-1}, 109.8 mmol L^{-1} or 164.6 mmol L^{-1} which corresponded to 10, 20, 30 g L^{-1} as a control treatment for osmotic potential. The effect of sucrose on the

Table 3. Physical appearance of sago palm leaf explants cultured in different sucrose and sorbitol combinations

Osmotic Potential (MPa)	Treatment	Sucrose (g)	Sorbitol (g)	Physical Appearance
-0.312	1	10	0	Explants turned brown while some were partly whitish.
	2	7.5	2.5	Explants turned brown, some were contaminated.
	3	5	5	Explants were whitish, some with signs of growth.
	4	2.5	7.5	Explants were whitish, some became brown/ contaminated.
	5	0	10	Explants turned brown or were contaminated.
-0.380	6	20	0	Explants turned brown, others were contaminated.
	7	15	5	Explants turned brown while only few were whitish.
	8	10	10	Explants turned brown while some were whitish.
	9	5	15	Explants turned brown while only few were whitish to greenish.
	10	0	20	Explants turned brown while only few were whitish.
-0.457	11	30	0	Explants turned brown while some were partly whitish.
	12	22.5	7.5	Explants were whitish to greenish, some with growth protrusion.
	13	15	15	Explants were whitish, some with growth protrusion.
	14	7.5	22.5	Explants turned brown while some were whitish.
	15	0	30	Explants turned brown, one was whitish with signs of growth.

growth of sago explants was also tested using different concentrations. Half-strength modified MS media supplemented with 7 mg L^{-1} 2,4-Dichlorophenoxyacetic acid and 0.1 mg L^{-1} kinetin at pH=6.8 was used. There were six treatments [(%w/v): 0% -control, 1%, 2%, 3%, 4%, 5% sucrose] with five replicates each. Each sucrose concentration has an equivalent molarity as follows: 0.0M, 0.03M, 0.06M, 0.09M, 0.12M and 0.15M. Osmotic potential of media for each concentration were calculated mathematically: -0.242 MPa, -0.312MPa, -0.385 MPa, -0.456MPa, -0.529MPa, -0.600MPa. Growth of explants was assessed by measuring the fresh mass.

Experimental Design

The experiments were replicated five times, where a culture bottle with one explant formed one replicate. A total of 75 explants were inoculated. A non-parametric measure (Kruskal-Wallis test) was used in analyzing data that do not meet the assumptions of Analysis of Variance (ANOVA) followed by pair wise comparison through Dunn's test statistic.

Results and discussion

Initiation of Growth

The presence of a cytokinin such as kinetin in the culture media is an important factor for shoot differentiation due to its role in cell division and inhibition of apical dominance (Bekheet and Saker, 1998). Being the most commonly used auxin, 2-4-D has a stimulatory effect on *in vitro* regeneration of plants Lakshamanan *et al.* (2002). In preliminary trials on growth initiation from sago palm shoot explants, no callus was formed but instead, there was direct shoot formation on explants cultured on modified MS medium supplemented with 7.0 mg L^{-1} 2,4-D in combination with 0.1 mg L^{-1} kinetin with 40% shoot formation frequency (Table 1). Thus, this media composition was used in the succeeding osmotic experiment. Figure 1 shows mean scores of explant growth while Fig 2 shows the different stages of direct shoot formation on a sago palm leaf explant in the growth initiation experiment.

Growth of explants under different concentrations of osmotic agents

The survivability of explants cultured in different osmotic concentrations using combinations of sucrose and sorbitol is

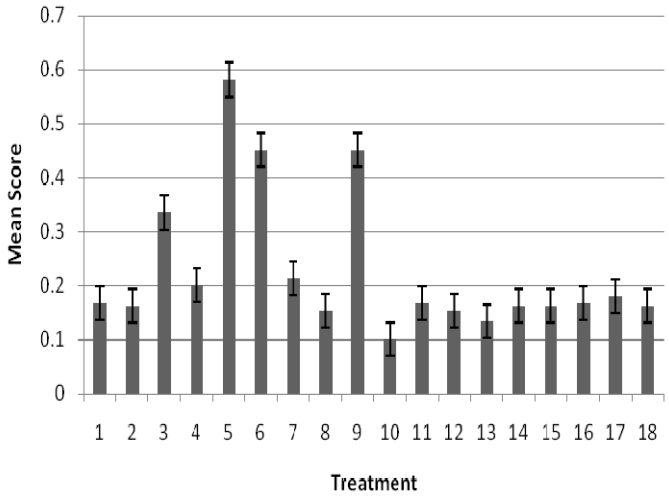

Fig 1. Mean scores of explant growth in the growth initiation experiment

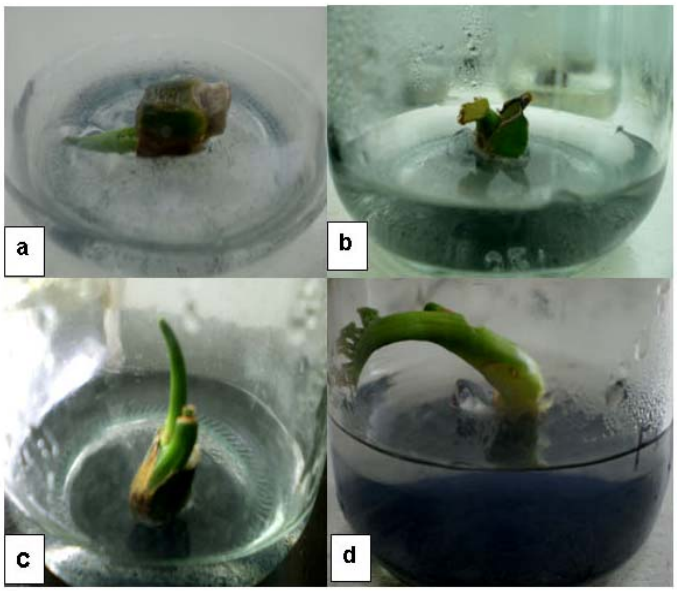

Fig 2. Explants with shoot formation cultured in half-strength MS media with 7 mg L^{-1} 2,4-D and 0.1 mg L-1 kinetin after: (**a**) 3 mo ~5cm (**b**) 4 mo ~7cm (**c**) 7 mo ~30cm, and; (**d**) 11 mo ~40 cm.

Table 4. Kruskal-Wallis test of mean weight increase of explants per osmotic treatment.*

Treatment	N	Mean Rank	Mean	
8	5	6.50	0.0620	a
9	3	10.33	0.0900	ab
10	3	12.67	0.1000	ab
14	3	14.67	0.1033	ab
11	5	17.00	0.1300	ab
1	3	19.00	0.1400	ab
7	3	22.17	0.1900	ab
4	4	27.50	0.2150	ab
3	4	29.38	0.2400	b
13	2	29.50	0.3300	ab
12	4	34.88	0.3475	bc

*Treatments are arranged from lowest to highest according to Mean Rank but with excluded treatments (T2, T5, T6, and T15) which have only one or missing data.

shown in Table 2. Percent survivability ranged from 20-100% except for cultures in treatments 5 and 6. Tissue browning or bacterial and fungal contamination caused the death of some cultures. The physical states of the explants were recorded and presented in Table 3. Table 4 reveals that the highest mean weight increase (0.35 g) of sago palm explants was obtained in medium containing 22.5 g sucrose and 7.5 g D-sorbitol (Treatment 12). Explants in Treatment 12 manifested meristematic protrusion and change of color from white to green. Some remained whitish while the others had poor growth qualities. The medium containing 15:15 g sucrose and sorbitol combination (Treatment 13) also promoted a high mean weight increase (0.33 g). These two treatments had low (or highly negative) osmotic potential of -0.457 MPa. Explants had less improvement in media containing 30 g sucrose but no sorbitol (Treatment 11) and also when sucrose concentration is lowered to 7.5 g combined with a high amount of sorbitol (Treatment 14). Cultures in Treatments 8, 9 and 10, containing lower sucrose concentrations (10, 5,0 g) combined with sorbitol (10, 15, 20) under -0.380 MPa generally, had the lowest weights gained. Meanwhile, explants cultured in media with 15 g surose and 5 g sorbitol (Treatment 7) produced a mean weight increase (0.19 g) higher than treatment 1 (0.14 g) but close to treatments 4 and 3 (0.215 g and 0.24 g, respectively) under -0.312 MPa. The Kruskal-Wallis test showed that there were significant differences among treatments in terms of mean weight increase. Pairwise comparison using Dunn's test statistic at alpha level=0.05 (95% confidence level) revealed significant differences between Treatments 8 and 13. Treatments 8 and 12 had significant differences when the alpha level was adjusted to 0.02 (80% confidence level). Therefore, the MS medium supplied with 22.5 g sucrose and 7.5 g sorbitol (-0.457 MPa) had significant effects on the increase of weight of sago palm explants, the osmotic concentration of which was deemed optimal for their growth. According to George (1993), osmotic potential of culture media influences the rate of growth, cell division or success of morphogenesis. This was confirmed by the results of this study wherein Treatments 12 and 13, possessing the lowest osmotic potential of media (-0.457 MPa), exhibited the greatest weight increases. In contrast, explants cultured in the intermediate osmotic potential (-0.380 MPa) obtained little increase in weight. At a high osmotic potential (-0.312 MPa), the explants' weights also increased markedly although not higher than Treatments 12 and 13. The weight increase of explants in the above mentioned media supplied with certain sucrose and sorbitol combination was possibly due to imbibition of water brought about by osmotic stress occurring in the explants. The sago palm explants may have also favored the lowest osmotic potential (-0.457 MPa) because they have relatively thick tissues which are capable of withstanding the high solute concentration. The sago palm seems to be a "sugary" plant. Hisajima *et al* (1995) reported the presence of sugars such as sucrose, glucose and fructose by paper chromatography. In view of its ecology, the sago palm is adapted to flooding even by saline water and to clayey soils with high organic matter content (Flach 1997). With this characteristic, *in vitro* culture of sago palm may also favor an environment with high solute concentration or low osmotic potential.

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