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The development of somatic embryos of sago palm (*Metroxylon sagu* Rottb.) on solid media[*)]

*Perkembangan embrio somatik tanaman sagu (*Metroxylon sagu *Rottb.) pada medium padat*

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Ringkasan

*Tanaman sagu (*Metroxylon sagu *Rottb.) biasanya diperbanyak secara vegetatif dengan tunas anakan. Namun, terbatasnya ketersediaan tunas anakan yang seragam merupakan hambatan utama dalam pembukaan perkebunan sagu. Teknologi kultur jaringan mempunyai potensi untuk perbanyakan klonal tanaman sagu unggul dalam skala besar. Kultur* in vitro *tanaman sagu telah dikembangkan melalui embriogenesis somatik. Kalus embriogenik yang berasal dari eksplan pucuk tunas anakan dikulturkan pada medium modifikasi Murashige dan Skoog (MMS) dengan sukrosa 30 g/L, Gelrite 2 g/L, arang aktif 1 g/L, 2,4-D 5 mg/L dan kinetin 0,1 mg/L untuk menginduksi embrio somatik. Kalus membentuk embrio somatik dalam waktu empat minggu. Dalam kultur berikutnya, dari kurang-lebih 0,3 g embrio fase globuler yang dikulturkan pada medium MMS dengan kinetin 1,0 mg/L, ABA 0,01 mg/L dan GA_3 0,1 mg/L menghasilkan 140 sampai 200 embrio somatik dengan fase perkembangan yang berbeda-beda. Embrio somatik dalam semua fase perkembangan dengan ukuran dan warna yang berbeda-beda ditemukan setiap saat dalam kultur. Di samping itu, embriogenesis somatik sekunder (berulang) juga terjadi dalam kultur sagu. Embrio somatik fase dewasa bila dipindah ke medium padat dengan garam makro setengah konsentrasi dan sukrosa pada konsentrasi 20 atau 30 g/L tanpa zat pengatur tumbuh akan menjadi planlet normal.*

Summary

Sago palm (*Metroxylon sagu* Rottb.) is usually propagated vegetatively by suckers. However, the limited availability of uniform suckers is a major obstacle in the establishment of cultivated sago plantations. Tissue culture has the potential for large-scale mass clonal propagation of superior genotypes of sago palm. *In vitro* culture of sago palm has been established through somatic embryogenesis. Embryogenic callus derived from shoot apical tissue of young suckers was cultured on a modified Murashige and Skoog (MMS) medium containing 30 g/L sucrose, 2 g/L Gelrite, 1 g/L activated charcoal, 5.0 mg/L 2,4-D, and 0.1 mg/L kinetin to induce somatic embryos. Callus clumps formed somatic embryos within four weeks. In the subsequent culture, approximately 0.3 g initial globular callus grown on MMS medium containing 1.0 mg/L kinetin, 0.01 mg/L ABA and 0.1 mg/L GA_3 produced 140 to 200 somatic embryos at different developmental stages four weeks later. All stages of developing embryos with different sizes and colors were present at any one time of culture. Secondary (repetitive) somatic embryogenesis was also found in the culture. Transferring of the mature stage of somatic embryos to solid media with

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half-strength macro salts and with sucrose at concentration of 20 or 30 g/L without growth regulators led to the development of normal plantlets.

[***Key words***: Embryo maturation, *Metroxylon sagu*, plantlet regeneration, sago palm, somatic embryogenesis].

Introduction

Sago palm (*Metroxylon sagu* Rottb.) is a staple food for Indonesian people especially in the eastern parts of the archipelago. Sago starch has been used for the production of noodles, white bread, high-fructose syrup, biodegradable filler in plastics, animal feed, adhesive, ethanol and many other derivative products (Flach, 1997). Sago palm has many advantages over other starch-producing crops especially for its higher yield (15-25 ton dry starch/ha/year), can grow along riverbanks and in swampy areas where are not suitable for other crops and no need replanted regularly (Flach, 1997).

Sago palm propagates vegetatively by suckers and generatively by seeds. Seed production is rare because the palms are commonly harvested by cutting the trees just before flowering. To establish large-scale plantations, the availability of uniform suckers is a major constraint (Jong, 1995). In addition, the weights of good-sized suckers which are 2 to 5 kg (Rostiwati *et al*., 1999) make them more difficult in their transportation.

Tissue culture has been conducted for the vegetative propagation of palms such as oil palm (Duval *et al*., 1993; Rival *et al*., 1997), coconut (Chan *et al*., 1998; Fernando & Gamage, 2000), and date palm (Omar & Novak, 1990; Huong *et al*., 1999). However, only few studies have been reported on tissue culture of sago palm (Hisajima *et al*., 1991; Tahardi *et al*., 2002).

Tissue culture of sago palm has been established through somatic embryogenesis, where early development of somatic embryos has been achieved by culturing shoot apical tissues as explants on a modified MS medium containing high concentrations of 2,4-D and 1 mg/L kinetin (Tahardi *et al*., 2002). Somatic embryo-genesis was chosen because of its potential for producing an abundant supply of uni-form superior planting materials (Handley, 1995). In this paper, the latest progress of our work in tissue culture of sago palm is reported especially in the induction and maturation of somatic embryos up to the formation of plantlets on solid media.

Materials and Methods

Plant materials and culture conditions

The research has been done at the Laboratory for Plant Cell Culture and Micropropagation, Indonesian Biotechnology Research Institute for Estate Crops, Bogor, West Java. Nodular embryogenic callus has been used as material for the research. The callus was initiated from shoot apical tissue of young suckers of field-grown sago palm in Parung district, West Java. The explants were cultured on a modified MS (Murashige & Skoog, 1962) medium (MMS) according to Tahardi *et al.* (2002) to initiate embryogenic callus. The pH of all media was adjusted to 5.7

before autoclaving at 121^0C and 1.0 kg/cm^2 for 20 min. All cultures were incubated in the culture room at the temperature of 25^0C under cool-white fluorescent lamps providing approximately 30 µmol photon/ m^2/s over a 14-h photoperiod.

Somatic embryo induction

The embryogenic callus was cultured on an MMS solid medium supplemented with 30 g/L sucrose, 1 g/L activated charcoal, 0.1 mg/L kinetin and different concentrations of 2,4-D: 0, 5 or 10 mg/L. Gelrite at 2 g/L was used as a gelling agent. Four clumps of callus were placed on a 40 mL solid medium in each culture jars with ten replicates. The cultures were placed in the culture room for four weeks. At the end of the experiments, the frequency of embryo formation was determined.

Somatic embryo maturation

Somatic embryos at early developmental stages, mostly globular, were cultured on a MMS solid medium containing half-strength macro salts, 2 g/L Gelrite, 30 g/L sucrose, 1 g/L activated charcoal, 0.01 mg/L ABA, 0.1 mg/L GA_3, and different cytokinin levels: 1, 2 and 4 mg/L BAP or kinetin. Four clumps of the embryos were placed on a 40 mL solid medium in each culture jars with five replicates. Total biomass fresh weight and the number of each embryo stages were observed at the initial and the end of the experiment (four weeks of culture).

Plantlet conversion

Mature somatic embryos mostly at cotyledonary stage were selected and cultured on an MMS solid medium with full- or half-strength macro salts and 20 or 30 g/L sucrose, without plant growth regulators. Four clumps of the embryos were cultured on a 40 mL solid medium in each culture jars with five replicates for four weeks. Total biomass fresh weight and the number of each embryo stages were observed at the initial of the experiment. After four weeks of culture fresh weight of total embryos, numbers of plantlets, and lengths of shoots and roots were determined.

Statistical analysis

Data were subjected to a two-way analysis of variance test (F test) after proper transformation if needed. Differences among treatment means were determined by Duncan's multiple range test at P=0.05.

Results and Discussion

Somatic embryo induction

Embryogenic callus of sago was white and soft consisted of many small nodular structures (Figure 1A). Embryogenic callus of sago started to form somatic embryos at ten days after culture. Some of white callus changed to mostly white yellowish or greenish with solid structures called

globular somatic embryos (Figure 1B). The concentration of 2,4-D with 5 mg/L 2,4-D affected significantly the formation of somatic embryos. All callus clumps produced somatic embryos on medium after four weeks of culture (Table 1). Addition of 2,4-D at 10 mg/L decreased the frequency of somatic embryo formation. On medium without 2,4-D only few of callus clumps formed embryos and the embryo formation had been delayed by more than one week.

Auxin 2,4-D in high concentration has commonly been used to initiate callus from explants of palm species including sago palm (Tahardi *et al*., 2002). However, the callus must be transferred to media with gradual decrease in 2,4-D level to the initial level such as in coconut (Chan *et al*., 1998; Fernando & Gamage, 2000) and in date palm (Huong *et al*., 1999) to induce somatic embryogenesis. In this study, it was found that 2,4-D at 5 mg/L combined with 0.1 mg/L kinetin promoted the frequency of somatic embryo induction (Table 1).

Somatic embryo maturation

There were approximately 80 somatic embryos mostly at globular stage when the cultures were started. Four weeks later, the total numbers of embryos increased to 140-200 embryos at different developmental stages on solid MMS media with 0.01 mg/L ABA, 0.1 mg/L GA_3 and BAP or kinetin at 1, 2, 4 mg/L (Figure 1C, Table 2). It means that new somatic embryos had been formed during the culture. These new embryos are called secondary (repetitive) somatic embryos. Some embryos grew further into later developmental stages (Figure 1D), while other embryos demonstrated the tendency of repetitive embryogenesis by budding off new globular embryos. Therefore, all stages of developing embryos with different sizes and colors were present at any one time over one passage of culture. Secondary embryogenesis was found in many other woody crops such as date palm (Huong *et al*., 1999) and tea (Akula & Dodd, 1998; Sumaryono *et al*., 2001).

During maturation process, somatic embryos of sago had changed in shape, size, and color. At early developmental stage the shape of the embryo of sago was rounded or globular with yellowish or greenish white in color then turned yellow or green reddish at later developmental stages (Figure 1C & 1D). The embryo shape had been changed

Table 1. Effect of 2,4-D on somatic embryo formation on solid MMS medium.
Tabel 1. Pengaruh 2,4-D terhadap pembentukan embrio somatik pada medium MMS padat.

Concentration of 2,4-D *Konsentrasi 2,4-D* (mg/L)	Somatic embryo formation *Pembentukan embrio somatik* (%)
0	10.0 c*
5	100.0 a
10	45.0 b

* Means followed by same letters are not significantly different.

* *Angka yang diikuti oleh huruf yang sama berarti tidak berbeda nyata.*

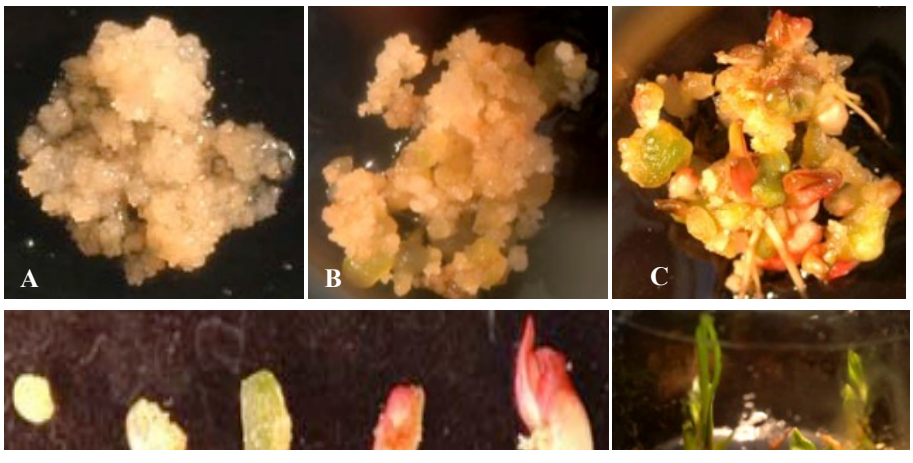

Figure 1. Somatic embryogenesis and plantlet formation of sago palm. (A) Embryogenic callus, (B) Early stage of somatic embryos, (C) A clump of somatic embryos at various developmental stages, (D) Somatic embryo development from globular to germinants, (E) Plantlets.

Gambar 1. Embriogenesis somatik dan pembentukan planlet tanaman sagu. (A) Kalus embriogenik, (B) Fase awal embrio somatik, (C) Kumpulan embrio somatik dalam berbagai fase perkembangan, (D) Perkembangan embrio somatik dari globuler sampai kecambah, (E) Planlet.

gradually from globular to heart-shape, torpedo, cotyledonary and early germinant (Figure 1D) followed the embryo development pattern described by Arnold *et al*. (2002). Unlike other crops, bright red and green colors were dominant in somatic embryos of sago. Cytokinin application affected significantly total number of somatic embryos. BAP at 2 and 4 mg/L and kinetin at 1 and 2 mg/L gave higher number of embryos than without cytokinin (Tabel 2). If only the number of somatic embryos at mature stage or germinated embryos is considered, kinetin at 1 mg/L was the best treatment for embryo maturation. This result is similar to embryo maturation of tea reported by Tahardi *et al*. (2000). Increasing the concentration of kinetin tended to decrease the number of mature embryos. This phenomenon may be caused by high availability of endogenous cytokinin so that the present of exogenous cytokinin could inhibit embryo maturation. Average weight of individual somatic embryos was ranged from 25 to 40 mg (Table 2).

Although more germinated embryos were found on the medium with 1 mg/L kinetin, the embryos were smaller than

Table 2. Effect of cytokinin on maturation of somatic embryos cultured on MMS medium with 0.01mg/L ABA.

Tabel 2. Pengaruh sitokinin terhadap maturasi embrio somatik yang dikulturkan pada medium MMS dengan ABA 0,01 mg/L.

Cytokinin *Sitokinin*	Concentration *Konsentrasi* (mg/L)	Number of somatic embryo *Jumlah embrio somatik*	Number of germinant *Jumlah kecambah*	Embryo weight *Berat embrio* (mg)
Control treatment *Kontrol perlakuan*		148.00 cd*	2.50 b	37.60 a
BAP	1	137.00 d	2.50 b	25.50 b
BAP	2	206.50 a	5.50 ab	40.10 a
BAP	4	195.80 ab	5.25 ab	33.20 ab
Kinetin	1	182.80 abc	8.75 a	27.60 b
Kinetin	2	190.00 abc	3.75 ab	28.00 b
Kinetin	4	152.50 bcd	0.50 b	26.30 b

* Means in the same column followed by the same letters are not significantly different.

* *Angka dalam kolom yang sama diikuti oleh huruf yang sama berarti tidak berbeda nyata.*

Table 3. Effect of macro salts and sucrose on plantlet conversion of sago on solid MMS medium without plant growth regulators.

Tabel 3. Pengaruh garam makro dan sukrosa terhadap konversi planlet sagu pada medium MMS padat tanpa zat pengatur tumbuh.

Treatment *Perlakuan*		Fresh weight of total embryos *Berat segar total embrio* (g)	Number of plantlets *Jumlah planlet*	Length of shoot *Panjang tunas* (mm)	Length of root *Panjang akar* (mm)
Macro salts *Garam makro*	Sucrose *Sukrosa* (g/L)				
Half-strength *Setengah kekuatan*	20	1.64 a*	13.00 a	3.83 a	11.97 a
	30	1.74 a	12.20 a	3.69 a	11.85 a
Full-strength *Kekuatan penuh*	20	0.99 b	8.00 b	2.70 b	2.63 b
	30	1.05 b	7.20 b	2.28 b	3.01 b

* Means in the same column followed by the same letters are not significantly different.

* *Angka dalam kolom yang sama diikuti oleh huruf yang sama berarti tidak berbeda nyata.*

those of on medium without cytokinin or with 2 mg/L BAP. BAP has been used for somatic embryos in several palms. In oil palm BAP at 5 and 10 µM were the best for embryo maturation on a solid medium that stimulated shoot development but halt root

development (Duval *et al*., 1993). The shoots of oil palm must be transferred to a rooting medium containing auxin. Aberlenc-Bertossi *et al*. (1999) found that the addition of BAP during embryo development of oil palm induced shoot apex differentiation thus increased germination rates by up to 70%. Similar results were found in date palm where embryo development was promoted by the combination of BAP and kinetin (Huong *et al*., 1999). Chan *et al*. (1998) achieved embryo maturation in coconut by decreasing the concentration of 2,4-D from 100 µM to 1 µM and the addition of BAP at 50 µM.

Plantlet conversion

Plantlet conversion was achieved on the medium with half-strength macros and sucrose at either 20 or 30 g/L (Table 3). On average there were 12-13 plantlets per jar on medium with half-strength macros. Half-strength macros also gave significantly higher fresh weight, lengths of shoot and root than those of full-strength macros (Table 3). Concentrations of sucrose 20 and 30 g/L did not affect fresh weight of embryos, number of plantlets, and lengths of shoot and root. Most plantlets had shoots and a root, sometimes more than one roots (Figure 1E). These plantlets were ready to be transferred on solid media for further growth. The same results were obtained in oil palm where most embryos developed into complete and normal plantlets after subculture onto half-strength MS devoid of plant growth regulators (Teixeira *et al*., 1995). Similar results were found in somatic embryogenesis of tea where the use of half-strength macro salts increased significantly the conversion of somatic embryos into plantlets (Tahardi *et al*., 2000). Devoid plant growth regulators have commonly been used for further development of somatic embryos into plantlets.

Conclusions

The best medium for induction of somatic embryos from embryogenic callus was MMS medium added with 30 g/L sucrose, 1 g/L activated charcoal, 0.1 mg/L kinetin and 5 mg/L 2,4-D. Kinetin at 1 mg/L promoted the maturation of somatic embryos on medium containing 0.01 mg/L ABA and 0.1 mg/L GA_3. During maturation somatic embryos underwent repetitive embryogenesis by budding off new globular embryos. The mature embryos grew into normal plantlets when cultured on medium with half-strength macro salts without plant growth regulators.

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