Somatic embryogenesis of sago palm (*Metroxylon sagu* Rottb.) through suspension culture technique

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Abstract. Mass production of sago palm (*Metroxylon sagu* Rottb.) through *in vitro* culture has not been fully explored. Suspension culture has a potency for scaling up the production of somatic embryos to support the mass production of the elite clones. This research aimed to determine the composition of cytokinins that efficiently induce somatic embryogenesis of sago palm in suspension cultures. A clump of friable calli derived from apical meristem culture was used as starting material. The calli were cultured on a modified MS medium with 1-5 mg/L kinetin and 1 mg/L TDZ. The results showed that the calli of sago palm grew well. The highest volume of calli at 5.67 mL was achieved at 1 mg/L kinetin + 1 mg/L TDZ. The lowest calli yield at 3.65 mL was produced at 5 mg/L kinetin + 1 mg/L TDZ. The highest fresh weight of sago palm biomass was achieved at 1 mg/L kinetin + 1 mg/L TDZ (4.2 g/flask). The fastest somatic embryo induction was at 7 weeks after culture in the best treatment (1 mg/L kinetin + 1 mg/L TDZ). At the medium supplemented with 1 mg/L kinetin + 1 mg/L TDZ produced on average 133.7 somatic embryos per flask.

1. Introduction

Sago palm (*Metroxylon sagu* Rottb.) is one of the potential crops for supporting food security and bioenergy in Southeast Asia particularly in Indonesia. Sago palms grow well in Eastern parts of Indonesia such as the Moluccas and Papua [1-3]. The area of sago palm in the world was estimated at 2.47 million ha in 1997 [4] then increased became 5.2 million ha in 2013 [3] and more than half grown in Indonesia. Sago starch has been used as a staple food especially in Eastern Indonesia for centuries [4].

Carbohydrate of sago palm could be used as raw materials for various industries including bioethanol [2, 5]. In the food industry, sago starch is a primary ingredient for noodles, cakes, and other food [3]. The starch can also be utilised as raw materials of adhesive gel, pharmaceutical, cosmetics, bioplastics, and paper [4, 6]. Although its industrial importance, large-scale sago palm plantations have not been developed yet. One of the problems was the unavailability of a large number of planting materials. Having proven well in some crops, a technique for somatic embryogenesis of sago palm has not been explored yet to produce mass clonal sago planting materials.

Somatic embryogenesis through liquid cultures have been developed for several plants i.e. yam (*Dioscorea alata* L.) ‘Pacala Duclos’ [7], black gram (*Vigna mungo* L.) Hepper [8], cucumber...
(Cucumis sativus L.) [9], Stevia rebaudiana [10], and Wasabia japonica [11]. Liquid culture is more efficient in using media, labour, and space than solid culture [11]. The liquid culture technique is implemented particularly for automatization, scaling up, increasing growth as well as uniformity of the cultured embryos [12, 13].

Somatic embryogenesis of some monocot plants has been well developed [14]. The uses of somatic embryogenesis techniques to produce clonal planting materials were reported for oil palm [15], coconut [16], date palm [17], peach palm [18] and sago palm [19]. The somatic embryogenesis of sago palm reported previously was utilized solid media culture. In liquid media culture technique, the composition of culture media especially the use of cytokinins played a vital role in somatic embryogenesis [20]. The aim of this research was to determine the composition of cytokinin to effectively induce somatic embryogenesis of sago palm in suspension culture.

2. Materials and Methods

2.1. Plant materials

The research was mostly conducted at the Laboratory of Plant Cell Culture and Micropropagation, the Indonesian Research Institute for Biotechnology and Bioindustry (IRIBB), Bogor, Indonesia. Histological analysis and observation were done at the Microtechnique Laboratory, Faculty of Biology - Bogor Agricultural University, and Research Centre for Biology - The Indonesian Institute of Sciences.

Friable calli were used as starting materials in this research (Figure 1A). The calli were initiated from young suckers of sago palm variety of Alitir originated from Merauke, Papua, Indonesia. The calli had been cultured several times before being used.

2.2. Media, treatment and culture conditions

The explants were cultured in a modified liquid MS [21] medium (MMS) as described by Tahardi [19]. The treatments were concentrations of kinetin: 0, 1, 3 and 5 mg/L, and each combined with 1 mg/L TDZ. The volume of the medium was adjusted to 30 ml/100 mL Erlenmeyer flask. The pH of all media was adjusted to 5.7 before being autoclaved at 121 °C and 1.0 kg/cm² for 20 min.

All cultures were placed on an orbital shaker at 80 rpm (Figure 1C) in the culture room at the temperature of 25 ± 1 °C under cool-white fluorescent lamps providing approximately 10 µmol photon/m²/s over a 12-h photoperiod for 10 weeks with one subculture.

2.3. Biomass growth and somatic embryo induction

Biomass growth was observed using CVS (Cell Volume after Sedimentation) method particularly to determine the growth trend of each treatment and the best growth. Measurement of CVS was done every week using the CVS tool (Figure 1B). Fresh weight of the biomass was observed at initial
culture and when harvested at the final culture using a digital balance in the laminar air flow hood. All cultures were observed the predominant colour after sieving process and transferred to the Petri dish. Observations were done in the laminar air flow hood. The number of somatic embryos was counted after sieving process and transferred on the Petri dish.

2.4. Histological study
Samples of somatic embryos were collected at the phase of elongated shape. The samples were fixed in 50% formaldehyde/acetic acid/alcohol (FAA) (v/v) The experiment was arranged using a completely randomized design with 10 replications. Data were subjected to one-way analysis of variance (ANOVA). Differences among treatment means were determined by Duncan’s multiple range test at P < 0.05. To improve the accuracy of the analysis, SPSS version 19 was used as a tool program for data analysis in accordance with the protocol of Johansen [22]. The samples were then transferred into 70% ethanol and dehydrated in an ethanol series. The samples were embedded in paraffin at 60 °C. Longitudinal sections were cut using a rotary microtome to 10 µm thickness. The sections were placed onto slides and stained with Johansen’s Safranin and Fast Green for 10 min [23]. The permanent slides were mounted in synthetic resin (Entelan®). Images were captured with an Optilab software camera attached to a Nikon Eclipse E100 microscope. The scales were projected in the same optical conditions.

2.5. Data analysis
The experiment was arranged using a completely randomized design with 10 replications. Data were subjected to one-way analysis of variance (ANOVA). Differences among treatment means were determined by Duncan’s multiple range test at P < 0.05. To improve the accuracy of the analysis, SPSS version 19 was used as a tool program for data analysis.

3. Results and Discussion

3.1. Biomass growth
The volume of sago palm biomass increased steadily until 10 weeks of culture (Figure 2). Fast increases in biomass started from three weeks, reached the peak at six weeks of cultures, and started to decrease afterwards (Figure 2). The fastest biomass growth was achieved at 1 mg/L kinetin + 1 mg/L TDZ, whereas the lowest was observed at 5 mg/L kinetin + 1 mg/L TDZ. At the end of 10 weeks of culture, the highest biomass volume at 5.67 mL/flask was achieved at 1 mg/L kinetin in combination with 1 mg/L TDZ, followed by 3 mg/L kinetin + 1 mg/L TDZ (5.29 mL/flask). Whereas the lowest biomass volume was 3.65 mL/flask observed at 5 mg/L kinetin in combination with 1 mg/L TDZ (Figure 2 and 3).

Within the first 3 weeks of culture, the increases of sago palm biomass volume were almost the same for all treatments at 0.9 to 1.7 mL/flask. The exponential growth rate of sago palm biomass occurred during the fourth to the sixth weeks of culture in the media supplemented with 1 mg/L kinetin plus 1 mg/L TDZ. These results clearly showed that the composition of the cytokinins was crucial in the biomass growth. Combination of 1 mg/L kinetin and 1 mg/L TDZ was the best among others.

After six weeks in the cultures, the sago palm biomass was subcultured to replenish the corresponding media compositions, marked as culture-2. Compared to the previous culture, the biomass growth rates of cultures-2 were much lower. In this case, the sources of energy in cultures-2 had been shared among the cells more for somatic embryo induction.

In general, the second suspension culture of sago palm showed that the biomass increases at all treatments were lower and almost the same and not significantly different. Therefore, the highest growth of sago suspension in the cultures-2 was achieved persistently in media with 1 mg/L kinetin in combination with 1 mg/L TDZ (Figure 3). The lowest biomass growth still occurred in media with 5 mg/L kinetin in combination with 1 mg/L TDZ (Figure 3). Figure 2, shows the trend of growth and development of sago palm suspension. The growth rate of initial culture (cultures-1 during the first
six weeks) was faster than during subculture (cultures-2). After the sago palm suspension has accumulated cellular biomass, the cells developed into aggregates for somatic embryo induction (developmental step). Cytokinin combination between 1 mg/L kinetin and 1 mg/L TDZ hastened somatic embryogenesis. Some papers reported that kinetin and TDZ application as single or in combination with other cytokinins induced somatic embryogenesis on plants like *Limonium sinensis* using 4.54 µM TDZ [24], *Coffea canephora* P ex Fr. using 0.93 µM kinetin [25], *Echinacea purpurea* L. using 2.5 µM TDZ [20]. Similar to that of Cinchona [26], the growth of sago palm biomass in suspension culture increased initially. Furthermore, when induction and maturation phases of somatic embryos occurred, it decreased the growth rate of biomass on sago palm [27].

**Figure 2.** Biomass growth curve of sago palm suspension in CVS from the initial culture until harvesting time at ten weeks.

Table 1 shows the fresh weights for all treatments at harvest. The highest fresh weight was 4.164 g/flask achieved at media with 1 mg/L kinetin in combination with 1 mg/L TDZ that appropriate with the achievement of highest biomass in CVS. Fresh weight was not always the same as CVS of sago palm biomass. Treatment media with 5 mg/L kinetin in combination with 1 mg/L TDZ resulted in the lowest biomass CVS (3.65 ml/flask) but it resulted a fresh weight higher than control treatment. The lowest fresh weight was 3.128 g/flask obtained at control treatment, whereas at media with 5 mg/L kinetin in combination with 1 mg/L TDZ resulted 3.739 g/flask although its biomass CVS was lower (3.65 mL/flask) than control treatment (4.09 mL/flask) (Table 1).

### 3.2. Predominant colour

In the initial culture, the callus was yellowish (Figure 1A). Furthermore, along with the growth and development of sago palm cultures, changes in colour, shape and size occurred. The colour change from yellowish to whithish occurred after four weeks of culture (culture-1). The callus was bigger and became more globular. The subculture stage (culture-2), produced the bigger callus with shape and colour clearly identified as proembryoids. In ten weeks when sago palm suspension was harvested, somatic embryos produced had developed to globular, elongated and scutellar shaped (Figure 6A-D).

The predominant light-yellow colour of somatic embryos occurred in almost all treatments at harvesting time. Somatic embryos which were brownish-yellow and bigger appeared in most treatments media with 3 mg/L kinetin in combination with 1 mg/L TDZ and media with 5 mg/L...
kinetin in combination with 1 mg/L TDZ (Table 1). Change in colour of somatic embryos from yellowish to reddish in the maturation stage occurred on solid media [28].

![Graph of biomass growth of sago palm suspension cultures in CVS in the first culture and the second culture (subculture).]

**Figure 3.** Biomass growth of sago palm suspension cultures in CVS in the first culture and the second culture (subculture).

| PGR’s Concentration (mg/L) | Fresh weight (g/flask) | Predominant colour |
|---------------------------|------------------------|--------------------|
| Kinetin TDZ               |                        |                    |
| 0 0                       | 3.128 ± 0.402          | Light-yellow       |
| 1 0                       | 3.234 ± 0.736          | Whitish-yellow     |
| 3 0                       | 3.737 ± 0.736          | Light-yellow       |
| 5 0                       | 3.736 ± 0.572          | Light-yellow       |
| 1 1                       | 4.164 ± 0.221          | Light-yellow       |
| 3 1                       | 3.796 ± 0.247          | Brownish-yellow    |
| 5 1                       | 3.739 ± 0.434          | Brownish-yellow    |

Values are mean ± SE from 10 replicates per treatment. Initial fresh weight: 0.3 g/flask

3.3. Somatic embryo induction

Sago palm callus suspension cultures started to form somatic embryos at 7 to 9 weeks after culture. Some of the white calli changed to mostly white yellowish or greenish with solid structures called globular somatic embryos when somatic embryogenesis was initiated clearly in the Erlenmeyer flasks.
The concentration of kinetin at 1 mg/L combined with 1 mg/L TDZ accelerated the initiation of somatic embryogenesis. All cultures formed somatic embryos at nine weeks after culture (Figure 4).

![Figure 4](image_url)

**Figure 4.** Average of time for somatic embryo initiation of sago palm suspension culture.

The highest number of somatic embryos induced (133.7 pcs) was achieved at media with 1 mg/L kinetin plus 1 mg/L TDZ and it was significantly different from K5 and the control but was not significantly different from media with 1 and 3 mg/L kinetin (as single), 3 mg/L kinetin + 1 mg/L TDZ and 5 mg/L kinetin + 1 mg/L TDZ. Whereas the lowest somatic embryo induction was obtained at control treatment (39.9 pcs). There were more mature somatic embryos obtained in the treatment combination between kinetin with TDZ than that of kinetin as a single treatment (Figure 5).

![Figure 5](image_url)

**Figure 5.** Number of somatic embryos induced on all treatments at ten weeks after culture.

Bars with different letters are significantly different according to Duncan’s multiple range test at $P < 0.05$. 

*Bars with different letters are significantly different according to Duncan’s multiple range test at $P < 0.05$.**
Somatic embryos were induced at all treatments. There were differences in time of somatic embryo formation and number of somatic embryos. Somatic embryos which were inducted from culture-1 during the six weeks cultures (older somatic embryos) were slightly different from somatic embryos from culture-2 (new somatic embryos) at the ten weeks. The differences in size (Figure 6C and 6D) and colour of the somatic embryos. The new somatic embryos were whitish and yellowish-white and the older somatic embryos were reddish, light-yellow or brownish-yellow (Figure 6B).

Addition of cytokinin increased somatic embryo induction whether as single PGR or in combination. Kinetin affected induction of somatic embryo when used as single treatment. Combination of kinetin and TDZ in proper concentrations increased the induction of somatic embryos [20, 29, 30]. This was confirmed also in sago palm cultures. In this study, it was found that 1 mg/L kinetin combined with 1 mg/L TDZ promoted the highest frequency of somatic embryo induction.

Figure 6. Somatic embryo of sago palm in suspension culture at 10 weeks after culture: (A) Biomass volume measurement by CVS; (B) Aggregate of somatic embryos; (C) Sieving for somatic embryo aggregates from suspension culture; (D) Somatic embryos after sieving; (E) Longitudinal section of somatic embryo at elongated stage. Scale bar = 1 cm (B and D). Length of a pen = 15.5 cm (C). SAM: Shoot Apical Meristem, RAM: Root Apical Meristem.

3.4. Histological study

The result of the histological analysis showed that cell structure was dense and homogeneous. The histological analysis displayed bipolar structures that comprised the top (shoot apical meristem, SAM) and bottom (root apical meristem, RAM) sides [31].

The top side that contains SAM was the shoot primordium and the bottom side that contains RAM was the root primordium (Figure 6E). A well-formed and dense structure indicated normal and good
somatic embryo with strong potency to develop into new individual plants [32]. The existence of SAM and RAM in somatic embryos will support the germination process of somatic embryos became normal plantlets having shoot and roots [33].

4. Conclusions

Somatic embryos of sago palm can be induced in a suspension culture. The best composition medium was 1 mg/L kinetin combined with 1 mg/L TDZ which resulted in the highest of biomass volume (5.67 mL/flask), biomass fresh weight (4.164 g/flask), and somatic embryo number (133.7 pcs/flask). During somatic embryo induction, the colour of calli has changed from mostly yellowish to light-yellow, whitish-yellow and brownish-yellow.

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