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EFFECT OF DIFFERENT SOURCES OF PLANT GROWTH REGULATOR ON THE INDUCTION AND DEVELOPMENT OF MANGOSTEEN SOMATIC EMBRYOS

Pengaruh Sumber Zat Pengatur Tumbuh Tanaman yang Berbeda pada Induksi dan Pengembangan Embrio Somatik Manggis

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ABSTRACT

Somatic embryogenesis is a technique for regenerating embryos derived from somatic cells of various plant species. This technique along with the utilization of plant growth regulator (PGR) might benefit for mass propagation and improvement of plant species through biotechnological tools. The study aimed to determine the effect of different plant growth regulators, namely 6-benzyladenine (BA) and thidiazuron (TDZ) on the embryogenic callus induction as well as casein hydrolysate and malt extract on the somatic embryo development of mangosteen. The explants used were *in vitro* young stems of mangosteen clone Leuwiliang. This study consisted of two experiments, namely induction of embryogenic callus and formation of somatic embryo. The first experiment was arranged as factorial in a completely randomized design with BA (0 and 0.7 mg l^{-1}) as the first factor and TDZ (0, 0.1, 0.5 and 1.0 mg l^{-1}) as the second factor. The second experiment consisted of four treatments, i.e. casein hydrolysate and malt extract at the rate of 500 and 1,000 mg l^{-1}. The results showed that the best medium for embryogenic callus induction was MS supplemented with 0.1 mg l^{-1} TDZ, which resulted semifriable calli. Casein hydrolysate and malt extract could not induce the formation of somatic embryos. After two times subcultures on the same MS medium supplemented with 0.5 mg l^{-1} TDZ and 0.7 mg l^{-1} BA, a total of 33.8 somatic embryos per explant was induced. The successful somatic embryogenesis would support mangosteen breeding and *in vitro* mass propagation program.

[***Keywords*****:** *Garcinia mangostana*, plant growth regulator, callus induction, somatic embryo]

ABSTRAK

Embriogenesis somatik merupakan proses pembentukan embrio dari sel somatik pada berbagai spesies tanaman. Teknik ini bermanfaat untuk perbanyakan benih tanaman secara massal dan perbaikan bahan tanaman dengan teknik rekayasa genetik. Penelitian bertujuan untuk mengetahui pengaruh beberapa zat pengatur tumbuh tanaman, yakni 6-bensiladenin (BA) dan thidiazuron (TDZ) terhadap induksi kalus embriogenik serta kasein hidrolisat dan ekstrak malt terhadap pembentukan embrio somatik manggis. Eksplan yang digunakan adalah batang muda in vitro *manggis klon Leuwiliang. Penelitian terdiri atas dua percobaan, yaitu induksi kalus embriogenik dan pembentukan embrio somatik. Percobaan induksi kalus embriogenik disusun secara faktorial dalam rancangan acak lengkap. Faktor pertama adalah BA (0 dan 0,7 mg l^{-1}) dan faktor kedua adalah TDZ (0; 0,1; 0,5 dan 1,0 mg l^{-1}). Percobaan pembentukan embrio somatik terdiri atas perlakuan kasein hidrolisat (500 dan 1.000 mg l^{-1}) dan ekstrak malt (500 dan 1.000 mg l^{-1}). Hasil penelitian menunjukkan bahwa media terbaik untuk induksi kalus embriogenik adalah MS yang diperkaya TDZ 0,1 mg l^{-1} yang menghasilkan kalus embriogenik dengan struktur agak remah. Kasein hidrolisat dan ekstrak malt tidak berpengaruh nyata dalam menginduksi pembentukan embrio somatik manggis. Setelah dua kali subkultur pada media yang sama, yaitu MS yang diperkaya TDZ 0,5 mg l^{-1} dan BA 0,7 mg l^{-1}, dihasilkan 33,8 embrio per eksplan. Keberhasilan embriogenesis somatik ini akan mendukung program pemuliaan tanaman dan perbanyakan benih massal manggis secara* in vitro.

[***Kata kunci*****:** *Garcinia mangostana*, zat pengatur tumbuh, induksi kalus, embrio somatik]

INTRODUCTION

Mangosteen (*Garcinia mangostana* L.) is an important tropical fruit which contributes to the Indonesian fruit export. The high demand for mangosteen has been difficult to be met due to the limited availability of mangosteen orchards, which are mostly consisted of intercept and backyard plants (Ministry of Agriculture Republic of Indonesia 2014).

To extend the mangosteen orchard, the availability of qualified seeds is required.

In vitro culture technology serves as an alternative technology to produce qualified mangosteen seeds. This technique consists of organogenesis and somatic embryogenesis. The accomplishment of the method is affected by several factors, namely explant source, basal media, growth regulator, organic chemicals, and regeneration system. The previous studies on mangosteen somatic embryogenesis resulted only low percentage (36–40%) of globular somatic embryos from young leaf explants (Elviana *et al.* 2011; Rohani *et al*. 2012; Rineksane *et al.* 2012). Thus, the mangosteen somatic embryo induction needs to be improved, using an appropriate source of explants, basal media, growth regulator and organic chemicals.

The most common growth regulator used for somatic embryo induction is auxin, particularly 2,4-D and picloram. Application of 2,4-D in somatic embryogenesis had been reported on guava (Fraga *et al*. 2012), chrysanthemum (Naing *et al*. 2013), tagara herbal plant (Chen *et al*. 2014a), and China rose (Chen *et al.* 2014b); whereas picloram had been applied on pineapple (Roostika 2012), grapes (Dai *et al*. 2015) and *Ledebouria* (Baskaran *et al*. 2016). Nevertheless, application of 2,4-D to induce mangosteen somatic embryos resulted scarcely regenerated calli (Te-chato 1998; Rohani *et al.* 2012). Besides auxin, thidiazuron (TDZ) and benzyladenine (BA) were used for inducing somatic embryogenesis on date palm (Aslam *et al*. 2011), *Camellia nitidissima* (Lu *et al.* 2013), olive (Mazri *et al.* 2013) and chestnut (Sezgin and Dumanoglu 2014). The combination of 0.7 mg l^{-1} TDZ and 0.7 mg l^{-1} BA on mangosteen culture produced 40% globular somatic embryos (Elviana *et al.* 2011).

The successful somatic embryogenesis was also determined by the use of organic compound, such as casein hydrolysate and malt extract. Al-Khayri (2011) demonstrated that application of 1,000 mg l^{-1} casein hydrolysate on date palm induced 29 somatic embryos per explant. George and de Klerk (2008) stated that casein hydrolysate acted as source of calcium, phosphate, several microelements, vitamin and 18 amino acids. Application of 1000 mg l^{-1} malt extract on murbey culture resulted 88% secondary somatic embryos (Agarwal *et al.* 2004).

The objective of this study was to determine the effect of 6-benzyladenine, thidiazuron, casein hydrolysate and malt extract on the induction and development of mangosteen somatic embryos.

MATERIALS AND METHODS

The research was conducted from May 2013 to April 2014 at Tissue Culture Laboratory of Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), Bogor, West Java. The histological study was conducted at Plant Growth and Development Laboratory, Faculty of Biology, Gadjah Mada University, Yogyakarta. This study was consisted of two experiments, namely embryogenic callus induction and somatic embryo formation.

Plant Materials

Plant material used in this study was mangosteen clone Leuwiliang, originated from Leuwiliang Sub-district, Bogor, West Java. The *in vitro* germinated mangosteen seeds were used as explant sources. The 10-week old shoots with 2 cm height were cut to 0.5 cm size and subcultured on embryogenic callus induction media.

Embryogenic Callus Induction

The experiment was arranged in factorial using a completely randomized design with eight replications. The first factor was BA (0 and 0.7 mg l^{-1}) and the second factor was TDZ (0, 0.1, 0.5 and 1.0 mg l^{-1}). Each treatment consisted of eight flasks as a replication, thus there were total 64 flasks for the whole experiments. Four young stem cuts were cultured in each flask.

The basal medium used was modified MS supplemented with 30 g l^{-1} sucrose, 2.5 gl^{-1} Phytagel, 300 mg l^{-1} glutamine and 100 mg l^{-1} PVP. The explants were incubated in continuous dark condition at temperature of 25 ± 1° C. Observations were carried out for 10 weeks on number of explants producing calli, callus weight, callus volume, callus visual structure and callus color. The callus volume was determined using a scoring system, namely 1 (callus covered <1/4 explants), 2 (callus covered 1/4–1/2 explants), 3 (callus covered 1/2–3/4 explants), and 4 (callus covered >3/4 explants).

Somatic Embryo Formation

The experiment was arranged as a completely randomized design with ten replications. The

treatments were single application of 500 and 1,000 mg l^{-1} casein hydrolysate, and 500 and 1,000 mg l^{-1} malt extract. Each treatment consisted of ten flasks with four callus aggregates initiated in each flask. The basal medium used was modified MS supplemented with 30 g l^{-1} sucrose, 2.5 g l^{-1} Phytagel, 0.1 mg l^{-1} TDZ, 300 mg l^{-1} glutamine and 100 mg l^{-1} PVP 100. The cultures were incubated in dark conditions at 25 ± 1° C temperature. The observations were conducted for 10 weeks on number of calli producing somatic embryos, number of embryos per explant and histological structure of the somatic embryos. The undeveloped embryogenic calli were then sub-cultured onto modified MS medium supplemented with 30 g l^{-1} sucrose, 2.5 g l^{-1} Phytagel, 0.5 mg l^{-1} TDZ, 0.7 mg l^{-1} BA, 300 mg l^{-1} glutamine and 100 mg l^{-1} PVP 100 and incubated in continuous dark conditions for 8 weeks, prior to the incubation in continuous light condition of about 1,000 lux for 8 weeks at 25 ± 1° C temperature.

Data Analysis

Data obtained from this study were analyzed using Statistical Analysis System (SAS 9.1) (SAS Institute Inc. 2004). The mean values were analyzed and compared using Duncan's multiple range test (DMRT) at $P = 0.05$.

RESULTS AND DISCUSSION

Embryogenic Callus Induction

The result showed that there was an interaction between BA and TDZ to the percentage of callus formation, callus weight and callus volume. The effect of TDZ addition either as single or in combination with BA was only observed two weeks after callus initiation. The effect was detected by swollen tissues on the cutting site of the tissues. In general, calli were produced three weeks after callus initiation.

Among the eight treatment combinations, seven formulated media were successfully inducing calli. Five media (0.1 mg l^{-1} TDZ and 0 mg l^{-1} BA; 0.1 mg l^{-1} TDZ and 0.7 mg l^{-1} BA; 0.5 mg l^{-1} TDZ and 0 mg l^{-1} BA; 1 mg l^{-1} TDZ and 0 mg l^{-1} BA; 1 mg l^{-1} TDZ and 0.7 mg l^{-1} BA) significantly induced more calli compared to other treatments (100% and 96.9%) (Fig. 1). The combination of 0.5 mg l^{-1} TDZ and 0.7 mg l^{-1} BA was unable to induce callus. Similar result was occurred on experiment using leaf explants (Joni 2014). It was suggested that MS supplemented with 0.5 mg l^{-1} TDZ and 0.7 mg l^{-1} BA altered the endogenous cytokinins of the tissue through homeostasis mechanisms on which the hormone was conjugated to glucoside and ribose thus reducing its effectiveness (Salisbury and Ross 1992; Taiz and Zeiger 2002). Furthermore, the explant response was

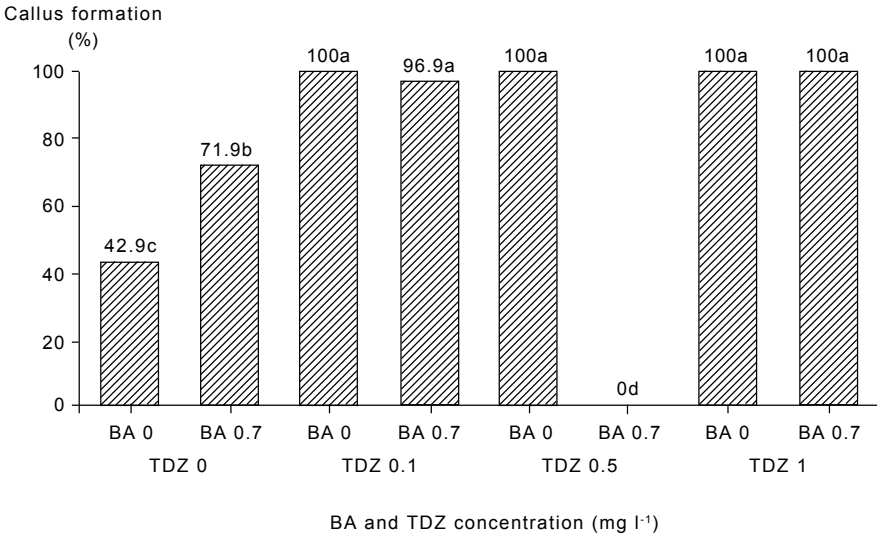

Fig.1. The effect of combined treatment of benzyladenine (BA) and thidiazuron (TDZ) on the callus formation of mangosteen explants at ten weeks after callus initiation. Means followed by the same letter is not significantly different according to Duncan's multiple range test at $P = 0.05$.

genotype dependent. This result is similar to that reported by Elviana *et al.* (2011) and Rohani *et al.* (2012) that such treatment could induce embryogenic calli by 3.3% and 20%, respectively.

The combination of 1 mg l^{-1} TDZ and 0.7 mg l^{-1} BA significantly produced higher callus fresh weight (0.36 g per explant) (Fig. 2). The largest callus volume (score 4) was obtained from treatment of TDZ 0.1 mg l^{-1}, combination of TDZ 0.1 mg l^{-1} and BA 0.7 mg l^{-1}, and combination of TDZ 1 mg l^{-1} and BA 0.7 mg l^{-1} (Fig. 3). It was showed that TDZ had an important role in callus induction and proliferation.

MS medium (MS0) also induced callus formation. The induced callus on MS0 medium was similar to callus formation on tobacco plant as reported by George and Sherrington (1984). The use of 0.7 mg l^{-1} BA also slightly induced shoots (data not shown). Callus structures on MS0 and 0.7 mg l^{-1} BA treatments were slightly different from those of other treatments, which were compact and had connected nodules (Fig.4).

The different callus structures and colors were also observed in each treatment. The callus structure could be differentiated into two groups, i.e. semi-

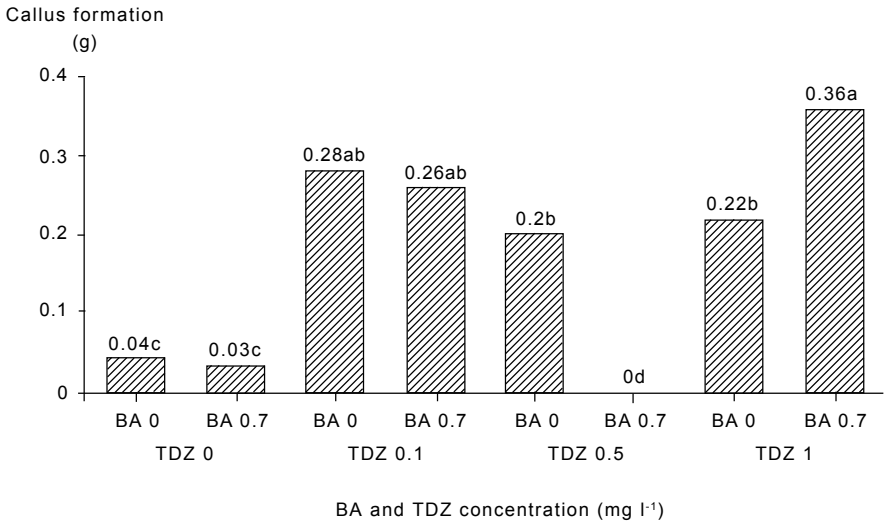

Fig. 2. The effect of combined treatment of benzyladenine (BA) and thidiazuron (TDZ) on the mangosteen callus weight at ten weeks after callus initiation. Means followed by the same letter is not significantly different according to Duncan's multiple range test at $P = 0.05$.

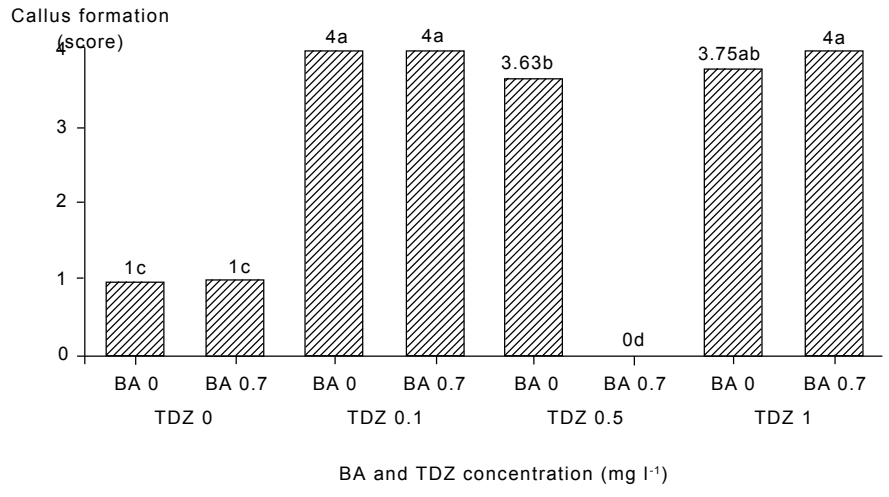

Fig. 3. The effect of combined treatment of benzyladenine (BA) and thidiazuron (TDZ) on the mangosteen callus volume at ten weeks after callus initiation. Means followed by the same letter is not significantly different according to Duncan's multiple range test at $P = 0.05$.

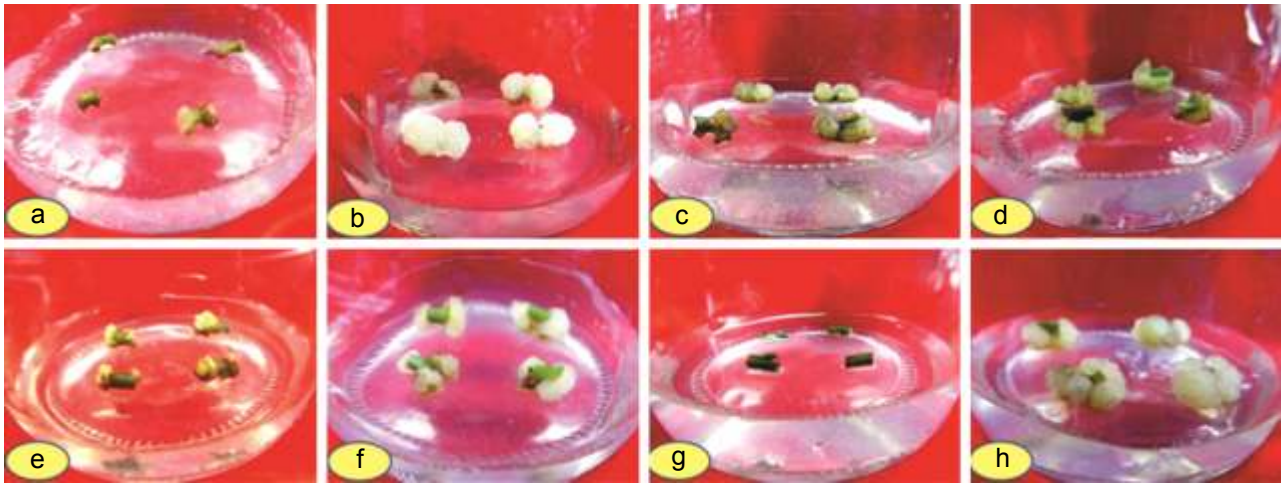

Fig. 4. The embryogenic calli of mangosteen derived from *in vitro* young stem explants at ten weeks after callus initiation in medium containing benzyladenine (BA) and thidiazuron (TDZ): (a) control, (b) 0.1 mg l^{-1} TDZ, (c) 0.5 mg l^{-1} TDZ, (d) 1.0 mg l^{-1} TDZ, (e) 0.7 mg l^{-1} BA, (f) 0.1 mg l^{-1} TDZ + 0.7 mg l^{-1} BA, (g) 0.5 mg l^{-1} TDZ + 0.7 mg l^{-1} BA, and (h) 0.1 mg l^{-1} TDZ + 0.7 mg l^{-1} BA.

friable and compact calli. The semifriable calli could be found in 0.1 mg l^{-1} TDZ treatment either as single treatment of 0.1 mg l^{-1} TDZ (resulted white calli) or combination of 0.1 mg l^{-1} TDZ and 0.7 mg l^{-1} BA; whereas the compact yellowish white calli could be found from other treatments (Table 1, Fig. 5). The semifriable calli potentially formed somatic embryos, which were initiated by the formation of globular structure. Based on this result, it was shown that TDZ at a concentration of 0.1 mg l^{-1} had an important role in formation of semifriable calli which potentially induced the mangosteen somatic embryos. Tuleja *et al.* (2014) reported that the use of TDZ at a concentration > 1 µM could stimulate callus, adventitious shoots and embryo somatic formation on *Pelargonium zonale.*

Table 1. The effects of combined treatment of benzyladenine (BA) and thidiazuron (TDZ) on mangosteen callus structure and color at ten weeks after callus initiation.

PGR (mg l^{-1})[1)]	Callus	
	Structure	Colour
MS0 (without PGR)	Compact	Yellowish white
TDZ 0.1	Semifriable	White
TDZ 0.5	Compact	Yellowish white
TDZ 1.0	Compact	Yellowish white
BA 0.7	Compact	Yellowish white
TDZ 0.1 + BA 0.7	Semifriable	White
TDZ 0.5 + BA 0.7	-	-
TDZ 1.0 + BA 0.7	Compact	Yellowish white

[1)]PGR = plant growth regulator

Somatic Embryo Formation

The experiment showed that casein hydrolysate and malt extract were unable to induce somatic embryos. Visual observation showed that the semifriable calli turned to be brownish after subculturing in new media. Treatments that caused calli browning were 500 and 1,000 mg l^{-1} malt extract (31.25% and 69.23%, respectively) (Fig. 6). The yellow semifriable calli became yellowish white compact calli after sub-culturing in new media containing casein hydrolysate or malt extract. After 2 weeks callus initiation, the texture and colour alteration of the calli were observed; the calli became watery, light brown and eventually the whole calli became dark brown and

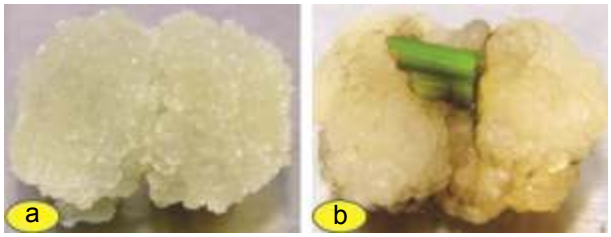

Fig. 5. Types of mangosteen calli: (a) semifriable calli and (b) compact calli.

died (Fig.7). It was speculated that the colour alteration might be due to the calli premature subculture into new media containing casein hydrolysate and malt extract, causing the calli were not ready for differentiating process.

Alteration from semifriable calli to compact calli was affected by calcium compound contained in the casein hydrolysate (George and de Klerk 2008). Calcium is an important element for cell middle lamella formation which had an intercellular joint function that is important in the formation of compact calli.

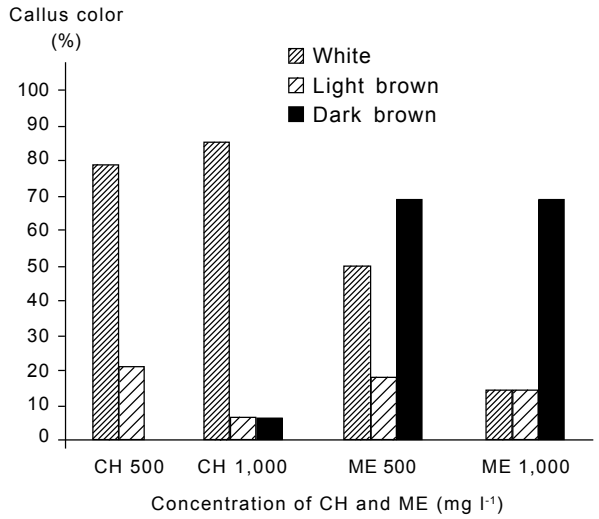

Fig. 6. The effect of casein hydrolysate (CH) and malt extract (ME) on the callus colour alteration of mangosteen derived from *in vitro* young stem at eight weeks after callus initiation.

Results of the experiment showed that casein hydrolysate and malt extract were not effective in inducing the mangosteen somatic embryos. A different result was reported by Gholami *et al.* (2013) that addition of 500 mg l^{-1} malt extract on orange somatic embryo media produced 36.50% globular embryo, 35.15% heart structure, 33.70% torpedo structure and 31.90% cotyledons. In oil palm somatic embryogenesis experiments, addition of 500 mg l^{-1} casein hydrolysate into the media produced 29.3 somatic embryos per explant (Al-Khayri 2011).

Because of the browning encountered, the survived white calli was then subcultured into new MS media enriched with 0.5 mg l^{-1} TDZ and 0.7 mg l^{-1} BA. In the first subculture experiments by growing the culture in continuous dark conditions, the calli turned to be more compact, greenish, with globular shape. In the second subculture, the cultures were incubated in continuous low light conditions and found that the globular structure eventually became green and formed shoot, as many as 33.8 embryos per explant (Fig. 8).

Based on the histological analysis (Fig. 9), there were morphological changes in every stage of

Fig. 7. The mangosteen callus colour alteration on casein hydrolysate and malt extract containing media at eight weeks after callus initiation: (a) yellowish white calli, (b) light brown calli and (c) dark brown dead calli.

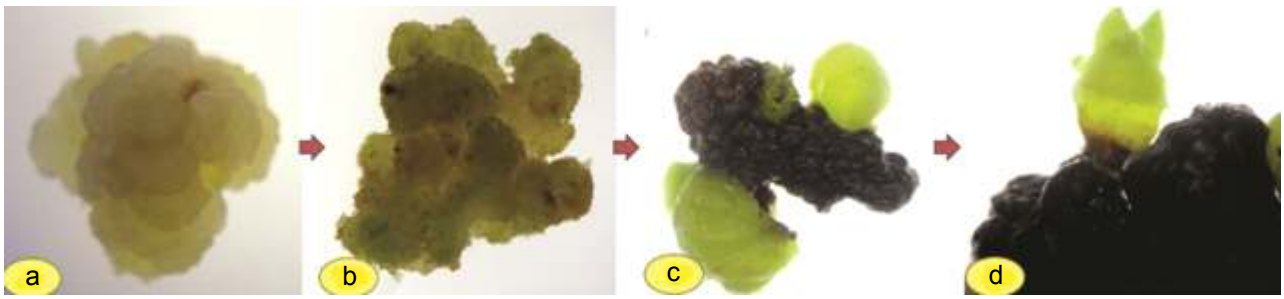

Fig. 8. The process of somatic embryogenesis formation on mangosteen: (a) semifriable calli, (b) greenish calli, (c) globular shape and (d) cotyledonary stage.

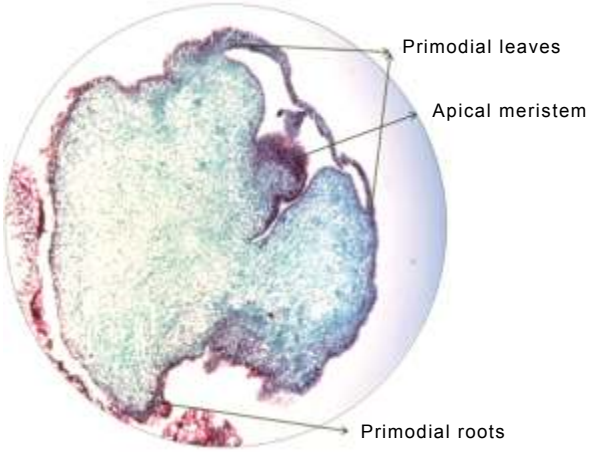

Fig. 9. Development of mangosteen embryo structure at the early stage of maturation based on histological study using Safranin 1% and Fast green 1%.

embryogenic callus development. As reported by Elviana *et al.* (2011), Rohani *et al.* (2012), and Rineksane *et al.* (2012), the mangosteen somatic embryo development was started with the globular stage and then directly went to the embryo maturation stage without experiencing the heart and torpedo stages. This process might reflect the anatomy of mangosteen seed which was different from that of other types of dicots plants by not showing differentiated cotyledons. In the early developmental stages, the whole cells demonstrated similar shapes and sizes. Further, on the globular and embryo maturation stages, the cells were separated from the mother cells. Somatic embryo is a bipolar structured individual with vascular tissues that are not connected to mother tissues.

CONCLUSION

The induction and development of mangosteen embryogenic calli appeared to be distinctive to the application of different plant growth regulators (PGR). Amongst the four kinds of PGR tested, only thidiazuron (TDZ) and 6-benzyladenine (BA) induced semi-friable structure embryogenic callus, whereas casein hydrolysate and malt extract did not. The best protocol for incuding mangostem somatic embryos is first growing the callus aggregates on the-MS medium supplemented with 0.1 mg l^{-1} TDZ to produce semi-friable calli, then the calli were subcutured twice on the media containing 0.5 mg l^{-1} TDZ and 0.7 mg l^{-1} BA to produce mangosteen cotyledon. The total of somatic embryos produced was 33.8 per explant. The success of producing mangosteen cotyledon via somatic embryogenesis reported in this study offers the possibility to support the mass propagation and to facilitate the fruit breeding program, either through somaclonal variation or genetic transformation.

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