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Comparison of Adventitious Shoot Formation of *Garcinia mangostana* via Embryogenesis and Direct Organogenesis

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ABSTRACT
The adventitious shoot of mangosteen can be obtained *in vitro* from seed and leaf explants. This research was conducted to study effects of Benzyl Amino Purine (BAP) treatments on the formation of adventitious shoot of mangosteen *in vitro* via embryogenesis and direct organogenesis. Explants for embryogenesis were taken from cotyledon, where as those for direct organogenesis were from red young leaf of seedling. The BAP treatment of embryogenesis and direct organogenesis were 0.0, 11.1, 22.2, 33.3 and 44.4 µM, medium supplemented with 3% sugar, 0.8% agar, 1.39 µM PVP. Each experiment was arranged in a completely randomized design with BAP concentrations as treatments. The result of embryogenesis showed that MS medium supplemented with BAP 22.2 µM BAP produced the best effects on the percentage of explants forming adventitious shoot (53.7%), number of shoots (3.3), length of shoot (1.7 cm) and time to form shoot (17.3 days). The result of direct organogenesis showed that MS medium supplemented with BAP at a concentration of 2.2 µM resulted in the highest percentage of explants that formed shoot (39.8%), number of shoot per explants (1.3 shoot), number of pair leaf (1.2) and means number of shoot with the length 1-5 mm (1.3 shoot), 6-10 mm (0.8 shoot) and >10 mm (0.3 shoot). Furthermore, at the concentration tested, the shortest time to form shoot was 80.7 days. This highly efficient protocol of embryogenesis and direct organogenesis of mangosteen is needed for the improvement of mangosteen such as in genetic transformation, mutation breeding methods and propagation of mangosteen.

Key words: Mangosteen, cotyledon, leaf explants, benzylaminopurine

INTRODUCTION

Mangosteen (*Garcinia mangostana* L.) is one of tropical fruit tree species cultivated mainly in South-east Asia. This fruit is known for its delicate exotic appeal hence it is referred as 'Queen of Tropical Fruit'. Mangosteen fruit has good prospect to be developed as export commodity and high economic value. Recently, Indonesia government has placed main priority to improve mangosteen as excellent export commodity (Qosim *et al*., 2011a).

Mangosteen is one of species in the family of Guttiferae/Clusiaceae that has 35 genera and more than 800 species from tropical regions. They are included nine genera with species of fruit trees. Five genera of the Guttiferae family have been cultivated mainly in South-east Asia (Verheij and Coronel, 1992). Mangosteen tree is suggested to be originated from

South-east Asia, possibly Indonesia (Yaacob and Tindall, 1995) and Malaysia (Richards, 1990a). Mangosteen is considered to be native to Indonesia, because it was found almost throughout the archipelagos in Indonesia especially in Sumatra, Borneo and Java Islands (Qosim *et al*., 2011a). The production center of mangosteen fruit are in West Sumatra, West Java, Central Java, East Java and Bali (Sobir and Poerwanto, 2007).

Mangosteen can be consumed as fresh fruit or processed food. Mangosteen fruits are mostly served as a dessert fruit. As processed food, mangosteen fruits are canned in heavy syrup, or as jam or crystallized, boiled pulp and seed with sugar, syrup puree and flavour for ice-cream or juice (Khalid and Rukayah, 1993). Besides its being used as food, mangosteen also has medicinal properties (Qosim *et al*., 2011b), because of the pericarp of mangosteen fruit contains xanthones compounds. More than 80 compounds have been isolated and characterized from the various parts of this plant (Obolskiy *et al*., 2009). Xanthones has been shown to have cytotoxic, antimicrobial, antifungal and antioxidant activities (Jung *et al*., 2006). In South-east Asia, A pericarp of mangosteen fruit has been used as traditional medicines for dysentery, wounds, skin infection, inflammation and diarrhea (Yaacob and Tindall, 1995).

Mangosteen trees have limitations i.e. slow growth rate of seedlings, long juvenile phase and lack of genetic variability (Qosim *et al*., 2011b). Mangosteen can be propagated from seeds apomicts obligate or agamospermy obligate. Mangosteen seeds are in the group of seed recalcitrant and formed obligate apomicts (Qosim *et al*., 2011b). The seeds come from nucellus cells and they are not resulted from pollination and fertilization (Richards, 1990b). Embryo of appears derived from somatic embryos, so it can be said that the propagation of mangosteen is a vegetative propagation. Mangosteen seed included in form apomixes adventitious embryony (Van Dijk and Damme, 2000).

The breeding of mangosteen, such as through *in vitro* mutation, genetic transformation and plants propagation needs high frequency plant regeneration system. The adventitious shoot of mangosteen come from embryo seeds (Goh *et al*., 1988); various explants from seedling grown *in vivo* (Goh *et al*., 1990); young and mature leaves explants from field grown trees (Goh *et al*., 1994), plant regeneration from nodular calli (Te-Chato and Lim, 1999). Embryogenesis is an adventitious shoot formation process from seed embryo, while direct organogenesis is an adventitious shoot formation process from leaf explants. However, high frequently embryogenesis and direct organogenesis of mangosteen has not been proposed by researcher.

The objective of this study was to compare high frequency of shoot regeneration via embryogenesis and direct organogenesis of mangosteen. The highly efficient protocol of embryogenesis and direct organogenesis is used to improve genetic transformation, mutation breeding methods and propagation of mangosteen.

MATERIALS AND METHODS

Plant materials and explants sources: The seed of mangosteen come was obtained from Purwakarta District-West Java, Indonesia. The embryogenesis used cotyledon as explants. Cotyledon was cleaned from aril (pulp) with a brush. The direct organogenesis used young red leaves derived from three-month-old seedlings in green house, as explants. Cotyledon and leaves as explants were sterilized by cleaning and soaking explants in 70% alcohol for 15 min and then soaking in solution HgCl 0.1% for 20 min and then rinsed by sterile water three times, respectively.

Embryogenesis and direct organogenesis: In embryogenesis, the cotyledon was cut into four segments. Indirect organogenesis, young red leaf explants (approximately 0.5×0.5 cm in size) were cut with midrib and then cultured on MS basal medium (20 mL). The BAP concentrations (0.0, 11.1, 22.2, 33.3, 44.4 μM) were used as treatments for embryogenesis and direct organogenesis. The leaf explants were grown in abaxial position. All media above were supplemented with 3% sugar, 0.8% agar and 1.39 μM PVP. The medium was adjusted to pH 5.7-5.8 with 0.1 M NaOH and then autoclaved at the pressure of 1.1 kg cm^2 and at temperature of 121°C for 20 min. The cultures were maintained at photoperiod of 16 h light per day and at temperature of 22°C under cool-white fluorescent light (28-30 μM sec^{-1} m^2).

Experiment design and statistical analysis data: Each experiment was arranged in a completely randomized design. Treatments were BAP concentrations and replicated twenty times (bottles). Each bottle consisted of four explants. All the data were analyzed statistically using F-test and the means were compared using the Duncan's Multiple Range Test (DMRT). Treatments were considered significant if $p \leq 0.05$. Data were analyzed using SAS Release 6.12 (SAS., 1996).

Histological observation: For histological analysis, samples from adventitious bud of 22.2 μM BAP via direct organogenesis were fixed in solution of Formaldehyde, Acetic acid and Alcohol (FAA) for 24 h, dehydrated through graded ethanol-xylol series (30-100% ethanol) and embedded in paraffin wax. Paraffin block containing embedded samples were sliced 10 μm thickness transverse sections by rotary microtome (Yamato RV-240). The sections were deparaffined in xylol, stained with safranin 1% and fast green 0.5% and examined under a microscope (Nikon HFX-DX).

RESULTS

This research showed that MS medium supplemented with BAP can influence adventitious shoots formation of mangosteen seed. Concentrations of BAP can influence the formation of adventitious shoot in embryogenesis and direct

Table 1: Adventitious shoots regeneration of mangosteen cotyledon segments via embryogenesis on MS medium treated with BAP

BAP treatment (µM)	No. of cultures	Explants producing shoots (%)	Means No. of shoots per explant	Time to produce shoots (days)	Mean height of shoots (cm)
00.0	20	4.4^{c}	0.2^{c}	19.0^{b}	0.2^{c}
11.1	20	31.2^{b}	1.2^{b}	19.7^{b}	1.6^{a}
22.2	20	53.7^{a}	3.3^{a}	17.3^{b}	1.7^{a}
33.3	20	26.2^{b}	1.2^{b}	23.6^{a}	1.1^{ab}
44.4	20	19.4^{bc}	0.8^{bc}	25.3^{a}	0.6^{bc}

Means within each column followed by the same letter are not significantly different at $p \leq 0.05$ according to DMRT, BAP: Benzylaminopurine

Table 2: Adventitious shoots regeneration of mangosteen via direct organogenesis on MS medium treated with BAP

BAP treatment (µM)	No. of cultures	Explants producing shoots (%)	Means No. of shoots per explants	Time to produce shoots (days)	Means number of shoots with the length (mm)		
					1-5	6-10	>10
0.0	19	0.0	0.0	0.0	0.0	0.0	0.0
11.1	19	22.4^{ab}	1.1^{a}	98.6^{ab}	0.4^{b}	0.6^{a}	0.1^{b}
22.2	19	39.8^{a}	1.3^{a}	80.7^{b}	1.3^{a}	0.8^{a}	0.3^{a}
33.3	20	20.0^{b}	0.6^{a}	109.4^{a}	0.5^{b}	0.4^{ab}	0.2^{ab}
44.4	20	21.3^{b}	0.8^{a}	105.0^{a}	0.8^{ab}	0.4^{ab}	0.1^{b}

Means within each column followed by the same letter are not significantly different at $p \leq 0.05$ according to DMRT, BAP: Benzylaminopurine

organogenesis. The seed explants of mangosteen cultured on MS medium without BAP produced less shoots. This result indicated that BAP was very important to stimulate the formation of adventitious shoot. Concentrations of BAP had influence on the formation of adventitious shoot, root and callus. Treatment of BAP at a concentration of 22.2 µM resulted in the highest percentage of adventitious shoot formation from seed explants (53.7%), number of shoot (3.3 shoot), length of shoots (1.7 cm) and time of shoot formation (17.3 days) (Table 1), while treatment of 11.1 µM of BAP produced the highest percentage of cultures that formed root and callus which were 0.6 and 12.5%, respectively (data not shown). Number of shoot varied from 2-15 shoot with the average was 3 shoot per explants. Mangosteen seed embryos appeared along the surface of the seed (Fig. 1a). So that, the mangosteen seeds are polyembrionic (Richards, 1990a). The multiple shoots grew on 22.2 µM BAP was shown in Fig. 1b.

The result of direct organogenesis showed that MS medium supplemented with BAP influenced the formation of adventitious bud from leaf explants. The leaf explants began to form shoots at 12 weeks. If the leaf explants response to the culture media, it remained green and formed adventitious shoots. The treatment of 22.2 µM BAP produced the highest percentage of cultures, which formed shoot (39.8%), number of shoot per explants (1.3 shoot) and number of pair leaf (1.2). Furthermore, it resulted in the fastest time to form shoot which was 80.7 days and the highest number of long shoot. The leaf explants of mangosteen cultured on MS medium without BAP (0,0 µM) did not produce shoots (Table 2). Adventitious bud grew from midrib of leaf (Fig. 2a). The shoots growing from leaf explants in the treatment of 22.2 µM BAP was shown in Fig. 2b.

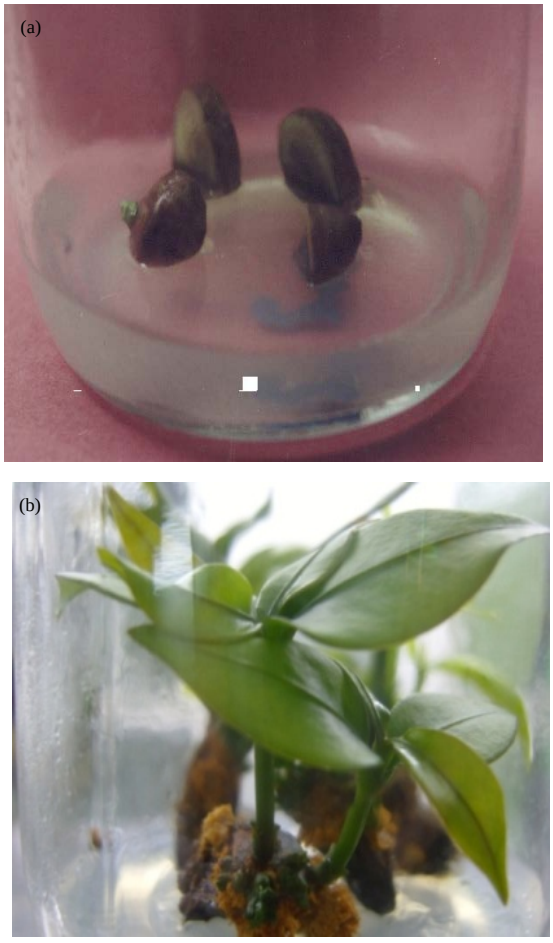

Fig. 1(a-b): (a) Adventitious bud from seed segment and (b) Multiple shoots from 22.2 µM BAP

Histological observation of direct organogenesis showed that the adventitious buds grew from midrib leaves possibly from vascular tissue (Fig. 3a), as meristematic tissue that exhibit epidermal cell became irregular. The mitotic activity of meristematic tissue, the cell was enlarge periclinal and anticlinal division occur the left and right of the central apical portion developed new meristem and then push the surface

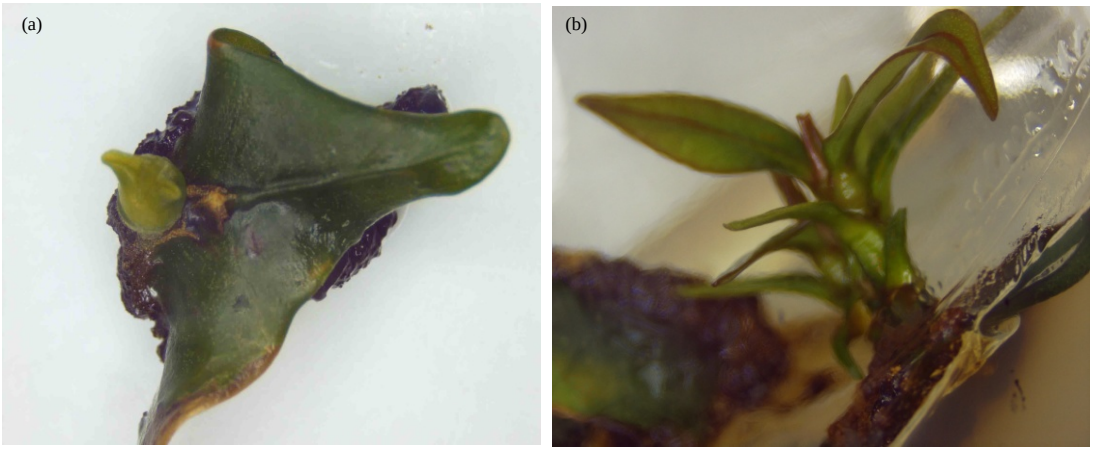

Fig. 2(a-b): (a) Adventitious buds appear from midrib leaf and (b) Adventitious shoot of treatment 22.2 µM BAP

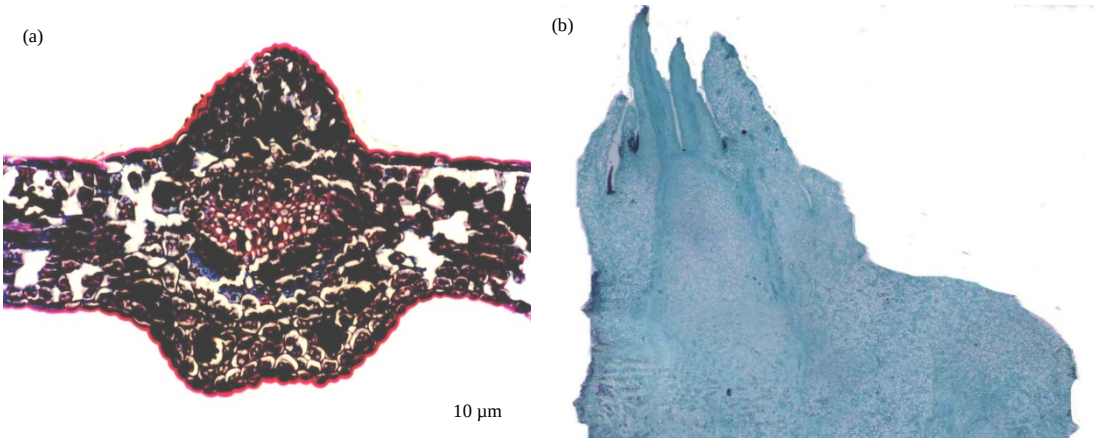

Fig. 3(a-b): (a) Transverse section of leaf mangosteen and (b) Shoot apical meristem

epidermal layer cause the epidermis to rupture. After that, the meristem dome became adventitious bud and produced leaf primordial. Peripheral pro cambium cells produced leaf primordium and then leaf bone from the procambium (Fig. 3b).

DISCUSSION

The adventitious shoot formation of mangosteen seed can be stimulated by BAP treatment on MS medium. In the previous studies, seed explants of mangosteen cultured on MS medium with 40 µM BAP concentration without NAA (Naphthalene acetic acid) produced more shoots (6.5 shoots) (Normah *et al*., 1990), whereas the use of NAA alone failed to stimulate shoot formation (Goh *et al*., 1988). In mangosteen, seed treatment is cut to produce six more shoot than cut three, besides better photoperiods eight hours from 12 h to induce adventitious shoot formation (Normah *et al*., 1990).

In mangosteen, experiments of plant regeneration using young green leaves from both seedlings and mature trees did not produce any shoots. Only callus tissues were produced but this was not found to be organogenic. The physiological and ontogenic age of the organ are important factors in influencing the behaviour of the explants in vitro. In general, the more juvenile the material, the easier the formation of organ *in vitro* (Goh *et al*., 1990). The juvenile red leaves from seedling grown in culture as well as 2-year-old field grown plant readily produced shoot, while young green leaves from both seedling and mature trees did not produce any shoot (Goh *et al*., 1988). Furthermore, the size of explants influence the formation epiphyllous buds. Halved leaf segments produced more buds than segments that cut into thirds and quarters, while whole leaves did not produce any shoot. Leaf segment derived from *in vitro* shoots showed a strong polarity of regeneration with shoot buds arising from the midrib near the distal cut end of leaf segment. Wounding of mid-rib, without complete severance and lamina of young red leaves triggered shoot but differentiation. It was observed that most of the buds were formed on the mid-rib, which was also the case in *Pistacia* (Barghchi and Alderson, 1982) and apple

(Liu *et al*., 1993) leaves tissues. The experiments on epiphyllous bud formation on young red leaf segments from seedlings are now being extended to the leaves of mature trees. The mid-rib leaves of mangosteen produced nodular calli (Qosim *et al*., 2013).

The adventitious bud formation in mangosteen leaf culture is controlled mainly by two factors, namely wounding of leaves and the availability of BA in the medium. The concentration of BA (Benzyl adenine) (20 μM) produced highest of the percentage of explants producing shoot buds (94%) and the number of shoot buds per leaf (7.7%) and high of shoot buds (2.1 mm) (Goh *et al*., 1994). A concentration of 4.45 μM BAP in WPM medium for shoots bud induction. Indirect organogenesis use of combination of 2.22 μM BAP and 2.27 μM TDZ on MS medium produce nodular calli basal medium WPM with 2.2 μM BAP concentration on medium WPM indicated the highest percentage of nodular callus formed shoot was 34.7%, average of numbers shoot per nodular calli was 7.8 shoots (Qosim *et al*., 2013). The use of BAP supra optimal (>44.4 μM) led to inhibit the formation and elongation of shoots. Consequence, shoots from cotyledon emerged more or less five months (Goh *et al*., 1988). The BAP application in tissue culture is used for regeneration of shoots in several plant species (Mondal *et al*., 1998).

In the plant bioassay system, BAP can regulate cell division, growth and differentiation of tissues and organs. BAP further states included in purine cytokinin group, which was instrumental in forming shoots as in peanuts (Victor *et al*., 1999). Cytokinin stimulates cell division and the transition in cell cycle from G_2 phase to mitosis phase (Salisbury and Ross, 1992). In cellular aspects of differentiated and organogenesis are regulated by the interaction between cytokinin and auxin. Auxin has on DNA replication (S phase), whereas the cytokinin influence mitotic division (George, 1993).

Cytokinin may play a role in protein synthesis, because: (1) cytokinin increase the speed of making RNA (tRNA, rRNA, mRNA) as it can increase the chromatin bound enzyme RNA polymerase, (2) cytokinin can increase the binding of tRNA aminoacyl on the ribosome that facilitate the introduction of codons, (3) cytokinin work by encouraging the formation of post-transcriptional polisom, so that mRNA can be translated not activated, (4) cytokinin in the regulation of protein synthesis is required in the formation of spindle function (Van Staden and Crouch, 1996).

Based on histological observation of direct organogenesis that adventitious buds grew from midrib leaves. The indirect organogenesis nodular calli grew from midrib leaves (Qosim *et al*., 2013). The midrib leaves possibly have vascular tissue, as meristematic tissue that exhibit epidermal cell became irregular depend on stimulate regulate growth substance. If midrib leaves stimulated by combination thidiazuron and benzylaminopurine epidermal cell irregular will be produced nodular calli, if midrib leaves stimulated by only benzylaminopurine epidermal cell irregular will be produced shoot.

CONCLUSIONS

The embryogenesis and direct organogenesis of *G. mangostana* L. can be enhanced by BAP treatment on MS medium. Optimum embryogenesis and direct organogenesis was achieved at the concentration of 22.2 μM BAP, which produced the highest number of adventitious shoot. However, time to produce shoot at direct organogenesis is longer than at embryogenesis. This highly efficient protocol of embryogenesis can be used to improve of *G. mangostana* L. such as mutation breeding methods, genetic transformation and plant propagation.

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