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Indirect Organogenesis and Histological Analysis of *Garcinia mangostana* L.

[1]W.A. Qosim, [2]R. Purwanto, [2]G.A. Wattimena and [3]Witjaksono
[1]Laboratory of Plant Breeding, Faculty of Agriculture, University of Padjadjaran,
Jl. Ir. Sukarno Km 21 Jatinangor Sumedang , 45363, Indonesia
[2]Departement of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University,
Campus IPB Darmaga Bogor, 16680, Indonesia
[3]Division of Botany, Indonesia Science Institute Jl. Ir. H. Juanda 22 Bogor, 16122, Indonesia

Abstract: The improvement of *Garcinia mangostana* L. through biotechnology approach needed an efficient plant regeneration system. The objective of this research was development of indirect organogenesis protocol for mangosteen such as effect of plant growth regulator for induction of nodular calli, plant regeneration and histological analysis of nodular calli and shoot bud. The treatments for induction nodular calli were concentration of BAP 2.2 and 4.4 μM while concentration of TDZ (thidiazuron) 1.14, 2.27 and 4.54 μM. Combination of BAP and TDZ concentration used as treatments. The medium of shoot proliferation was Woody Plant Medium (WPM) with concentration of BAP (0.0; 1.1; 2.2; 3.3; 4.4) μM as treatments. All experiments were arranged completely randomized design and analyzed data using F-test and the means among each treatment was separately by Duncan's Multiple Range Test (DMRT). A result showed that combination of 2.22 and 2.27 μM TDZ on MS medium produce nodular calli basal medium WPM with 2.2 μM BAP concentration indicated the highest percentage of nodular callus formed shoot was 34.7%, average of numbers shoot per nodular calli was 7.8 shoots, average of time formed shoot was 94.5 days and number of shoot length 1-5 mm was 11.06, shoot length 6-10 mm was 2.61 and shoot length >10 mm was 0.61. The highly efficient protocol of indirect organogenesis suggested in this study can be used to mutation breeding methods and propagation of *Garcinia mangostana* L.

Key words: Mangosteen, nodular calli, shoots

INRODUCTION

Mangosteen (*Garcinia mangostana* L.) is one of tropical fruit tree species known for its delicate exotic appeal hence the tree is referred to as 'Queen of Tropical Fruit' (Wieble, 1993). Mangosteen fruits has high economic value, thus has good prospect to be developed into an excellent export commodity. Recently, the government of Indonesia has placed high priority to develop mangosteen for export commodity. Available statistical data showed that the production of mangosteen fruit was 72, 634 metric tons in 2006 to 105, 558 metric tons in 2009 was increase of almost 45% in four years. However, production of mangosteen was decrease only 97, 484 metric tons in 2011. Export volume of mangosteen was 5, 697 in metric tons 2006 to 11 388 ha in 2010, an increase almost 100 % in five years (MoA, 2012).

Mangosteen fruit can be consumed fresh and also as processed food. In South-east Asia, the fruit pericarp of mangosteen has been used traditionally as medicine for inflammation, diarrhea, dysentery, wounds and skin infection (Yaacob and Tindall, 1995). Recently, researchers have carried out intensive studies about compound of fruit pericarp of mangosteen. On a fruit pericarp of mangosteen has been found xanthones compound and has medical properties. Xanthones have functions as antifungal, antimicrobial, antioxidant and cytotoxic activities (Jung *et al*., 2006).

Mangosteen trees have four limitations. First, slow growth rate of seedlings, this is due to the lack of root system in which both formation root hairs are few and low capacity of CO_2 capture leaves (Wieble, 1993), so seed two year old seedlings can not reach the high 15 cm (Lan, 1984). Second, long juvenile phase, the first mangosteen fruit reached 10-15 years old after planting (Wieble, 1993). Third, genetic variability of mangosteen is narrow (Wieble, 1993). Mangosteen seeds are formed apomictically (obligate apomicts) and in the group seed recalcitrant. The seed is not resulted from pollination and fertilization (Richards, 1990) but the fruit comes from nucellus cells. Embryo appears derived from surface of seeds, so it can be said that propagation of mangosteen is vegetative propagation.

Correspponding Author: Warid Ali Qosim, Laboratory of Plant Breeding, Faculty of Agriculture, University of Padjadjaran, Jl. Ir. Sukarno Km 21 Jatinangor Sumedang, 45363, Indonesia Tel/Fax: +62 22 7793316

The improvement of mangosteen with biotechnology approach such as genetic transformation, induction mutation *in vitro*, somaclonal variation and plants propagation needed an efficient plant regeneration system. The micropropagation of mangosteen from seeds (Goh *et al*., 1988); various explants from seedling grown *in vivo* (Goh *et al*., 1990); young and mature leaves from field grown trees (Goh *et al*., 1994) have been used and plant regeneration via nodular calli (Sompong and Lim, 1999). Indirect organogenesis is process of adventitious shoot formation through callus stage. However, protocol of indirect organogenesis of mangosteen not established yet and histological analysis not proposed yet by researcher.

The objective of this research was development of indirect organogenesis of mangosteen protocol such as effect of BAP and TDZ combination treatment for induction of nodular calli and effect of BAP treatment for shoot proliferation and histological analysis of nodular calli and shoot bud of mangosteen.

MATERIALS AND METHODS

Plant materials: Young reddish mangosteen leaves derived from three-month-old seedlings were used as explants to induce nodular calli. The explants sterilization process carried out by cleaning the leaves and soaked explants in 70% alcohol for 15 min and then soaked in 0.1% HgCl solution for 20 min and then rising three times with sterile destilated water, respectively.

Induction of nodular calli: Young leaf explants (apromiximately 0.5×0.5 cm in size) were sectioned with midrib (Fig. 1a) and then incubated on MS basal medium supplemented with 3% sucrose, 0.8% pure agar and 1.39 μM PVP. The explants were incubated on abaxial position. Plant growth regulators were BAP with concentrations of 2.2 and 4.4 μM while the concentration of TDZ with 1.14, 2.27 and 4.54 μM, combination of BAP and TDZ concentration used as treatments. The explants with nodular calli were counted and subcultured interval four weeks in the same medium. After four weeks of the percentage of explants forming nodular calli, number of nodular calli per explant, shape and color of nodular calli and time of formation nodular calli were recorded.

Shoot proliferation: The treatments of shoot proliferation were concentration of BAP (0.0; 1.11; 2.22; 3.33; 4.44 μM) on WPM (woody plant medium) medium. The all media above were supplemented with 3% white sugar, 0.8% pure agar, 1.39 μM PVP. The pH of both the medium adjusted to pH 5.7-5.8 with 0.1 M KOH and then autoclave on

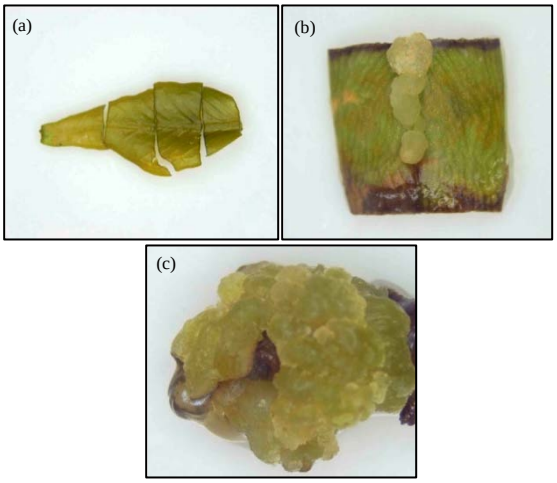

Fig. 1(a-c): Young leaf as (a) Explants, (b) Nodular calli appear from midrib leaf and (c) Nodular calli of treatment combination 2.22 μM BAP and 2.27 μM TDZ

temperature 121°C dan pressure 1.1 kg cm^2 for 20 min. The cultures were maintained under condition 16 h under cool-white light (30-40 μmol $m^{-2}sec^{-1}$) at temperature 22°C.

Histological observation: For histological analysis samples from transverse sections of nodular calli and shoot bud were studied using a paraffin method (Johansen, 1940). Nodular calli and shoot bud were fixed in FAA (formaldehyde, acetic acid and alcohol) solution for 24 h and dehydrated in Johansen series solution with graded series alcohol (30, 50, 75 and 95%) transitioning to Tertiary Butyl Alcohol (TBA) 50% and paraffin resin 50% for 2-24 h. Hence, it has been infiltrated with tertiary butyl alcohol and paraffin resin into blocks and incubation 58°C for 12 h. A 10 μm thick transverse section of samples were sliced by rotary microtome (Yamato RV-240) and then coloring in an alcoholic-xylol series with 1% safranin and 0.5% fastgreen (Sass, 1951) and examined under a microscope (Nikon HFX-DX).

Experiment design and statistical analysis data: All experiments were completely randomized design and experiment consisted of four explants per culture bottles. Each treatment replicated twenty times (bottles). All data were be transformated and statistically analyzed using F-test and the means among each treatment were separately by Duncan's Multiple Range Test (DMRT). Software SAS Release 6.12 (SAS Institute, 1996) was used for all statistical analysis and value of $p<0.05$ was considered significant.

RESULTS

Leaf explants of *G. mangostana* were incubated on solid MS medium with varying level combination BAP and TDZ for induction of nodular calli. After two weeks leaf explants enlarged and formed nodular calli from midrib leaf and petioles laminae (Fig. 1b), after three weeks nodular calli were enlarged and different in size. The tip of the leaf lamina usually dried. The characteristic of nodular calli were compact, yellow-greenish white and also green (Fig. 1c). The highest percentage of explants produced nodular calli were 79.41% and the number of nodular calli per explants were 3.6 derived from 2.22 μM BAP and 2.27 μM TDZ combination treatment while the lowest percentage of explants produced nodular calli were only 38.2% and the number of nodular calli per explants were 1.9 derived from 4.4 μM BAP and 4.54 μM TDZ. There were no sign of nodular calli formation when explants were cultured in MS media without BAP or TDZ (data not shown). Time of appeared nodular calli on MS medium was within 22-28 days with average 24.5 days after cultures (Table 1). The rate of growth of nodular calli increased from the second week of culture to four weeks. However, after six weeks nodular calli declined.

After four weeks, nodular calli incubated on media WPM medium supplemented various BAP treatment indicated that there were sign nodular calli formed shoot bud. The color of nodular calli were yellow-greenish white can be regenerated produced shoots. This result showed that WPM basal medium with 2.2 μM BAP concentration produced the highest percentage of nodular calli produced shoot were 34.7%, number of shoots per nodular calli reached 20 shoots with average of number shoot were 7.8 shoot per nodular calli while treatment of 4.4 μM BAP concentration lowest produced shoot. There were no sign of nodular calli produced shoots when cultured in WPM medium without BAP treatment. The ability of nodular calli regenerated shoots promoted by BAP treatment. Time of produced varied from 95-105 days. Time of produced shoots rapidly 94.5 days on 2.2 μM BAP concentration while BAP treatment 4.4 μM produced shoots longer than other ones (Table 2).

Based on histological analysis showed that nodular calli appeared from wounding of midrib leaf explants. The epidermis cell has a regular, well ordered appearance on all three sides. The more central mesophyll tissue was compact and has little intercellular space. After 3 days on hormone containing induction medium, the cell of epistomatic surface have become irregular in size and shape, resulting in an uneven leaf surface. The presence of meristemoid was easily observed after 12-18 days and is denoted by the darkly stained nuclei in the group of small cells clustered. The meristematic rapidly increase in size. Meristematic growth may also occur laterally along the plane of the explants surface. The meshopyll cells surrounding the central vascular tissue were compact and exhibit very little intercellular, epidermal cell become irregular. Small group meristemoid cell mainly formed clusterly under the epidermal cell. The meristematic cells enlarged both perpendicularly and parallel to form nodular calli. The mitotic activity of the epidermal cell will lose much of their regularity and become iregularity in length forming small cell clusters. The activity of meristematic cell begin to form meristematic domes that eventually protrude from the surface of the explants (Fig. 2 and 3a). The development of structure dome involve incorporation

Table 1: Effect of various combinations BAP and TDZ to induce nodular calli of mangosteen derived from leaf explants on MS medium

Combination treatment (μM)					
BAP	TDZ	No. of culture	Explants produced nodular calli (%)±SE	Mean No. of nodular calli per explants±SE	Time of produced nodular calli (days)±SE
2.2	1.14	17	72.3±17.9^{a}	2.4±1.1^{b}	25.7±4.8^{ab}
2.2	2.27	20	79.4±12.6^{a}	3.6±1.0^{a}	24.5±4.8^{b}
2.2	4.54	19	64.5±22.5^{ab}	3.3±1.0^{a}	26.4±5.8^{ab}
4.4	1.14	16	64.1±18.6^{ab}	2.1±1.2^{b}	23.1±4.1^{c}
4.4	2.27	16	51.6±21.9^{bc}	2.1±1.0^{b}	22.5±9.9^{c}
4.4	4.54	17	38.2±22.7^{c}	1.9±0.9^{b}	27.7±3.7^{a}

Means followed by the same letter are not significally different (p = 0.05) by Duncans Multiple Range Test (DMRT), SE: Standard error

Table 2: Effect of BAP treatments to adventitious shoots development from nodular calli of mangosteen in WPM medium (indirect organogenesis)

					Mean No. of shoots at length (mm)		
BAP treatment (μM)	No. of culture	Produced shoots (%)	Mean No. of shoots per nodular calli	Time of produced shoots (days)	1-5	6-10	>10
0.0	19	0.0	0.0	0.0	0.0	0.0	0.00
1.1	17	25.0^{a}	5.9^{ab}	105.7^{ab}	6.6^{b}	1.6^{ab}	0.20^{b}
2.2	18	34.7^{ab}	7.8^{a}	94.5^{b}	11.1^{a}	2.6^{a}	0.60^{a}
3.3	19	13.3^{b}	4.5^{b}	110.6^{a}	4.3^{bc}	1.4^{ab}	0.15^{b}
4.4	18	15.9^{b}	3.0^{bc}	105.0^{ab}	4.1^{b}	0.7^{bc}	0.00^{b}

Means followed by the same letter are not significally different (p = 0.05) by Duncans Multiple Range Test (DMRT)

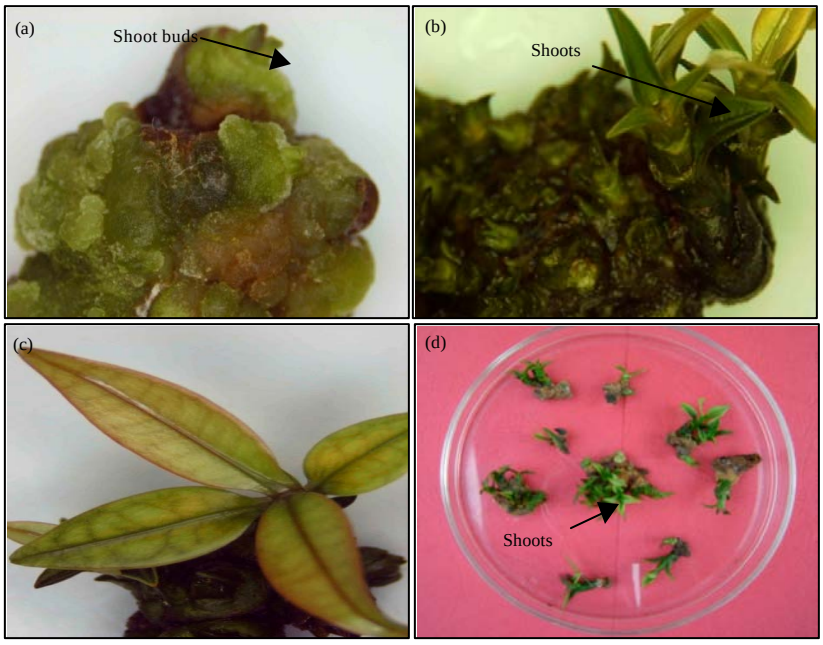

Fig. 2(a-d): Nodular callus form (a) Shoot buds, (b) Multiple shoots bud, (c) Shoot of 2.22 µM BAP and 2.27µM TDZ treatment and (d) Multiple shoots of mangosteen

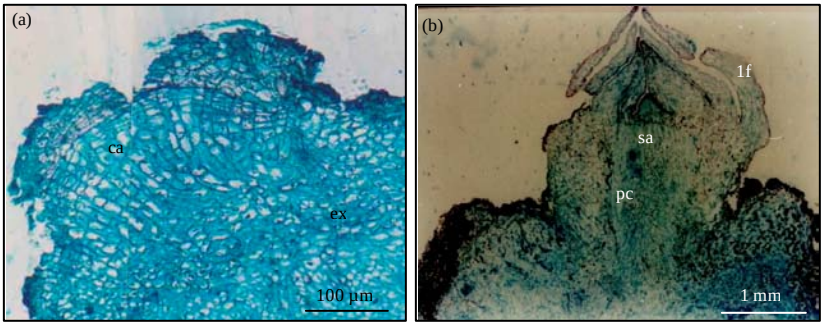

Fig. 3(a-b): Transverse section of (a) Nodular callus and (b) Shoot bud of 2.2 µM BAP treatment, lf: Leaf primordial, sa: Shoot apex, pc: Procambium

epidermis cell, the meristematic domes have been observed to push through the surface epidermal layer, causing the epidermis to rupture. The numerous anticlinal division in the epidermis just to the left of the center of the dome and periclinal division just to the right of the center of the dome. Both periclinal and anticlinal division occured the right and left of the central apical portion of the newly developed meristem, allowing for the successive production of leaf primordia. The shoot apex continues to grow giving rise to numerous leaf primordia and culminating in the formation shoot bud. Leaf primordia supported by peripheral procambium cells and then the procambium will be a bone of the leaf (Fig. 3b).

DISCUSSION

Indirect organogenesis offer promise for rapid multiplication plant, mutation breeding, genetic transformation and somaclonal variation. In this study, how were the effect of BAP and TDZ combination treatment for induction nodular calli and shoot regeneration. The result showed that induction of nodular calli promoted by BAP and TDZ combination treatment. Sompong and Lim (1999) stated that the using combination BA and TDZ induced nodular calli while the using only BA or TDZ alone could not induced of nodular

calli. This result more best than result research Sompong and Lim (1999) that induction of nodular calli of mangosteen on solid MS medium with 2.22 μM BAP and 2.27 μM TDZ produced nodular calli 34% after three weeks. The use TDZ (0.1-0.5) μM on MS medium was effective for adventitious bud differention in *G. indica* and *G. cambogia* (Kalia *et al*., 2012). In the some cases, TDZ alone was reported to be an effective growth regulator for shoot proliferation and embryo somatic (Yasseen, 1984). TDZ is one of penhylurea group that plays an important role in enhancing the endogenous cytokinin biosynthesis and accumulation of purine (Capella *et al*., 1983). In addition, the TDZ can subtitute adenine in the formation of calli culture and micropropagation in woody plant species (Lu, 1993). The use of TDZ was affective in wide variety of plant species for induction of somatic embryogenesis (Malik and Saxena 1992), shoot organogenesis (Liu *et al*., 1993). Amongs the cytokinin, TDZ was also found most effective for shoot proliferation in tissue culture, the use of TDZ at low concentrations (<μM) can induce buds on woody plant species whose seeds are recalcitrant (Huetteman and Preece, 1993) and was also found superior than other cytokinin for induction multiple shoot (Thomas and Puthur, 2004). Fiola *et al.* (1990) stated that the use of TDZ less 1 μM can be induced calli, adventitious shoots or somatic embryos on woody plants. Guo *et al*. (2012) stated that shoot regeneration of *Saussurea involucrate* on medium MS containing 0.5 μM TDZ produced 15.6 shoots derived from leaf explants. The presence of TDZ in combination with BAP and NAA on regeneration *Vigna mungo* L. *in vitro* significantly increase formation of multiple shoot bud (Acharjee *et al*., 2012).

The shoot proliferation stimuled by cytokinin. The result showed that shoot proliferation promoted by BAP treatment. The using only BAP alone, nodular calli could not regenerated shoots (Table 2). In fact, nodular calli could not produced shoots when cultured in WPM medium without BAP treatment. The cytokinin plays an important role in plant bioassay system. First, the group produces a purine derivative BAP instrumental in inducing physiological responses such as regulation of cell division, differentiation of tissues and organs as well as chlorophyll biosynthesis. Secondly, the group that produces synthetic derivative phenylurea such as thidiazuron plays an important role in enhancing the endogenous cytokinin biosynthesis and accumulation (Victor *et al*., 1999). In tobacco, TDZ has been found more active than BAP or zeatin in stimulating calli formation and morphogenesis (George, 1993). TDZ has been reported to be the most active cytokinin like substance for shoot induction.

According to Smopong and Lim (1999), induction of shoot primordia of mangosteen from nodular calli can be done on MS or WPM medium. Furthermore, WPM medium with a concentration of 4.45 μM BAP for induction shoot bud and inhibit the elongation of shoots. The use of BAP supra optimal (> 44.4 μM) to inhibit the formation and elongation of shoots. BAP application in tissue culture is used for regeneration of shoots in several plant species (Mondal *et al*., 1998). Victor *et al.* (1999) suggested that the plant biossay system, BAP can regulate cell division, growth and differentiation of tissues and organs. The function of cytokinin can promote cell division by accelerating the transition from G_2 phase to mitosis in cell cycle phase (Salisbury and Ross, 1992). In tissue culture or organ, cellular aspects of differentiated and organogenesis is controlled by the interaction between cytokinin and auxin, auxin effect on DNA replication (S phase), whereas the cytokinin effect in mitotic division (George, 1993).

CONCLUSION

The optimum protocol of indirect organogenesis of *G. mangostana* consist of two stage: (i) Induction of nodular calli using MS medium with BAP treatment combination 2.22 μM BAP dan 2.27 μM TDZ can induce nodular calli derived from leaf explants, (ii) Shoot regeneration was 2.2 μM WPM with BAP treatment. The highly efficient protocol of indirect organogenesis can be used to improvement of *Garcinia mangostana L.* such as mutation breeding methods, genetic transformation, variation somaclonal and propagation.

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