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Full Length Research Paper

In vitro development of embryogenic calli and embryogenic stages in suspension cultures of mangosteen (*Garcinia mangostana* L.)

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In this study, the potential of uncoated and coated mangosteen seed explants in forming embryogenic callus were examined in the basal Linsmaier and Skoog medium supplemented with different auxins at various concentrations. Among the highest percentage of callus response (93.3%) was obtained when uncoated seed explants were cultured in basal LS medium containing 8 mg/L 2,4-D. Combining of cytokinins and 2,4-D to improve embryogenicity of calli showed that among the highest percentage of callus response (80%) and the lowest percentage of callus browning (53.53%) with yellowish and compact nodular calli were obtained on MS medum supplemented with 8 mg/L 2,4-D and 0.1 mg/L BAP. Addition of glutamine into MS medium containing 8 and 0.1 mg/L BAP performed that glutamine did not increase the growth of calli, however texture (friable) and color callus (yellowish) was improved. The growth and multiplication of cells in suspension cultures showed that the cells were able to divide and proliferate even though cultured in half strength MS liquid medium without 2,4-D. After six months of culture, the heart embryogenic stage was obtained only on medium supplemented with 1 mg/L BAP. The globular and torpedo embryogenic stages were obtained on media supplemented with 1, 3 and 9 mg/L TDZ after five months of culture.

Key words: Mangosteen, embryogenic callus, suspension culture, *in vitro.*

INTRODUCTION

Mangosteen (*Garcinia mangostana* L.) is a popular tropical fruit with increasing demand for fulfilling the needs of consumers in health product. The antioxidant ingredient in pericarp such as xanthone, alpha and beta mangosteen is utilized as anti cancer agents. Mangosteen can be consumed as table fruits or processed as juice, health drink and health supplement. Its fragrant edible fruit has delicious and sweet sour taste, for that this fruit is known as 'the queen of the fruits' (Osman and Milan, 2006; Pedraza-Chaverri et al., 2008). For the purpose of meeting the industrial demand of the fruit, there have been various difficulties in securing the availability of mangosteens as commercial plantations. One of the problems regarding to the establishment of mangosteen plantation is obtaining seedlings throughout the year. Mass productions of seedlings are achievable through micropropagation of undifferentiated plant tissue for organogenesis and somatic embryogenesis.

Plantlets of mangosteen derived from *in vitro* organogenesis had been set up using seeds (Normah et

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Abbreviations: IAA, Indole acetic acid; **IBA**, indole butyric acid; **NAA**, naphthylene acetic acid; **BAP,** benzyl adenine purin; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **TDZ**, thidiazuron; **MS**, Murashige and Skoog; **LS**, Linsmaier and Skoog.

al., 1995); young leaves *in vitro* (Lakhsmanan et al., 1997; Sirchi et al., 2008), field-grown seedlings and young leaves derived from mature trees (Goh et al., 1990). Research in inducing embryogenic callus of mangosteen has been done (Te-chato et al., 1995). However, the result showed that there were no somatic embryos produced. Presently, there is no report of mangosteen suspension culture of cells and the formation of somatic embryos. Hence, this study are attempted to develop embryogenic calli and embryogenic stages in suspension cultures of mangosteen.

The objectives of the study were to determine the effect of explants types, glutamine, different types and concentrations of auxin and cytokinin on callus formation, to induce and multiply cell growth in liquid culture and to determine the effect of BAP and TDZ on the formation of advanced embryogenic stages.

MATERIALS AND METHODS

Explants material and aseptic preparation

Explants used in this study were Mangosteen seeds. Seeds were cleaned to rid of the fleshy and juicy part of the fruits. They were washed thoroughly under running water for 5 min and immersed in 2 g/L detergent solution for 5 min. Then the seeds were immersed in 5 g/L of Benomyl solution for 3 h and rinsed three times with distilled water. Seeds were divided into two groups, the coated and uncoated seeds (coats were removed in an aseptic environment). All seeds were sterilized with 10 and 5% Clorox® (Sodium Hypochlorite 5.25%) for 5 and 10 min, respectively, and each seed was cut into four sections in an aseptic environment.

Development of embryogenic calli

The effect of auxins on callus induction

The basic culture medium used in this study was the Linsmaier and Skoog (LS) medium containing 30 g/L sucrose, 0.2% activated charcoal, 2.5 g/L of phytagel agar supplemented with various auxins (IAA, IBA, 2,4-D, and NAA) at various concentration of 0, 0.5, 1, 2, 4, 8, 16, 32 mg/L. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH and 0.1 N HCl. All cultures were kept at 25 ± 2°C, under white fluorescent light at a photon flux intensity of 13.5 to 18 µmol m^{-2} s^{-1} under a photoperiod of 16 h light.

The effect of 2,4 D and cytokinins on callus induction

In the present study, only the uncoated seed segments were used as explants. Two types of basic medium was used in this study, which were Murashige and Skoog (MS) and LS media containing 30 g/L sucrose, supplemented with various cytokinins at different concentration levels (0 and 0,1 mg/L BAP; 0, 0.1, 1, 2, 3, 4, 5 mg/L TDZ ; 0, 0.1 mg/L Kinetin) either alone or in combination with different concentrations of 2,4-D (0, 8, 16, 32 and 64 mg/L).

The effect of glutamine on embryogenic callus induction

The uncoated seed segments were cultured on half strength MS medium containing 8 mg/L 2,4-D and 0.1 mg/L BAP supplemented with different concentrations of glutamine (0, 100, 200, 300, 400 and 500 mg/L).

Development of embryogenic stages in suspension cultures

Cell multiplication

For the initiation of suspension cultures, approximately 0.1 g six month-old calli from half strength MS medium supplemented with 8 mg/L 2,4 D; 0.1 mg/L BAP and 500 mg/L glutamine were carefully separated with sterile forceps and transferred onto half strength MS liquid medium containing 0, 2, 4 and 8 mg/L 2,4-D. The flasks were incubated on a gyratory shaker (100 rpm) at 25 ± 2°C under white fluorescent light at a photon flux intensity of 13.5 to 18 µmol m^{-2} s^{-1} under a photoperiod of 16 h light.

The effect of BAP on the development of embryogenic stages

The effect of BAP on suspended cell growth was carried out by transferring the cells from D1 Murashige and Skoog (MS0) liquid medium to half strength MS supplemented with 500 mg/L casein hydrolysate, 30 g/L sucrose and different concentrations of BAP (1, 3 and 9 mg/L).

The effect of TDZ on the development of embryogenic stages

The explants were uncoated seed segments of mangosteen which was cultured on half strength MS solid medium supplemented with 8 mg/L 2,4-D, 0.1 mg/L TDZ and 500 mg/L glutamine. The cultures were subcultured monthly for 10 months. The one gram friable calli formed then were transferred onto half strength MS liquid medium supplemented with 8 mg/L 2,4-D, 0.1 mg/L TDZ and 500 mg/L glutamine. The liquid cultures were subcultured monthly for 3 months. The suspension cultures were sieved through a nylon screen 250 and 100 µm, respectively. The effect of TDZ on cell suspension growth was carried out by transferring the sieved suspension cells from half strength MS liquid medium supplemented with 0.1 mg/L TDZ and 500 mg/L glutamine to half strength MS liquid medium supplemented with 500 mg/L casein hydrolysate with different concentrations of TDZ (1, 3 and 9 mg/L).

Statistical analysis

The statistical analysis of auxins effect on callus induction was arranged in a Randomized Complete Block Design (RCBD). Each treatment was replicated 9 times. The parameters observed were the percentage of explants producing calli, calli texture and size, and percentage of browning callus after 8 weeks of culture.

The statistical analysis of effect 2,4-D and cytokinins on callus induction was arranged in a RCBD. Each treatment was replicated 15 times. The parameters observed were percentage of explant forming calli, calli texture and size, and browning after 8 weeks of culture.

The statistical analysis of effect glutamine on embryogenic callus induction was arranged in a randomized complete block design (RCBD). Each treatment was replicated 10 times. The parameters observed in this study were the initiation of calli in growth after 8 weeks of culture (based on the scoring callus as shown in Table 1) and the detection of embryogenic calli. Six month old calli obtained from this experiment were assessed for their capacity to form somatic embryos using the double staining method (Jain and Gupta, 2005). All data were analyzed using ANOVA and treatment means were compared using Duncan new multiple range test (DNMRT) at $\alpha = 5\%$.

Table 1. Callus scoring criteria.

Callus score	Diameter of callus (mm)	Growth of callus
1	0	No callus formation
2	> 0 – 5	Some
3	> 5 – 10	Moderate
4	> 10 – 15	Abundant
5	> 15	Most Abundant

The statistical analysis of cell multiplication, BAP and TDZ effect on embryogenic stages were arranged in a completely randomized design (CRD). Each treatment was replicated 3 times. The parameter observed in this study was including the morphology of cells by examining under an inverted microscope. Daily cell growth was not observed due to slow growth of cells and limited amount of cell cultures. Differences in cell growth and cell development among treatments were qualitatively observed under an inverted microscope Zess® Axiovert 135. Cell viability was tested using the Fluorescein diacetate method (FDA) (Widholm, 1972).

RESULTS AND DISCUSSION

Development of embryogenic calli

The effect of auxins on callus induction

The effects of auxins, IAA, IBA, 2,4-D and NAA in the induction of callus from mangosteen seed segments has not been reported before. In the present study, both coated and uncoated seed segments started to initiate callus on the wounded side of the segments within 7 to 14 days of culture.

Percentage of explants forming callus

The percentage of explants forming callus shows significant responses to the concentration of auxins that were added into media. The results in the present study show that interaction between auxin types and concentrations affected the percentage of explants producing callus as shown in Figure 1. In general, all concentrations of 2,4-D produced the higher percentage of callus response compared to IAA, IBA and NAA. The increasing concentrations of IAA, IBA and NAA up to 4 mg/L decreased the percentage of callus response. The percentage of callus response produced by adding IAA, IBA and NAA became higher after the concentration 8 mg/L was applied and continued increasing until 32 mg/L. In contrast, percentage of callus response increased with increasing 2,4-D concentration until 8 mg/L 2,4-D. However, concentrations higher than 8 mg/L 2,4-D resulted in the decreased percentage of callus response as shown in Figure 1.

Previous work by Ribnicky et al. (1996) on carrot hypocotyls-derived callus cultures showed that 2,4-D, IAA and NAA enhanced callus growth. Ribnicky et al. (1996) have demonstrated that metabolism and associated activity of 2,4-D in culture are very different from that of applied IAA and NAA. 2,4-D is a relatively stable compound due to its persistent in carrot culture (Michalzuk et al., 1992) and strong auxin due to its effect in callus proliferation (Ammirato, 1985). Exogenous free 2,4-D is not easily conjugated by carrot hypocotyls. This resulted in the abundant proliferation of callus in the 2,4-D-treated-carrot hypocotyls. In contrast, both NAA and IAA conjugates with aspartate which accumulated in much lower total amounts than 2,4-D. The amount of free NAA and IAA was much less than the amount of conjugated NAA and IAA. In terms of accumulation and conjugation, NAA appears to be intermediate between IAA and 2,4-D. The developmental effects of NAA treatment were also intermediate between those of 2,4-D and IAA. NAA-treated hypocotyls proliferates callus and became organogenic, producing roots, shoots and embryos (Ribnicky et al., 1996).

The lowest effect of IAA on the growth of callus here can also be explained by the previous work by Ribnicky et al. (1996) which conclude that exogenous IAA activate the IAA conjugation system. The IAA conjugates depleted the free IAA which resulted ineffectiveness of the free IAA concentration to induce much callus proliferation. The second is the relatively weak effectiveness of IAA in certain long-term tissue cultures since IAA will be degraded in the presence of both light and nutrient salt. The third is the exogenous IAA suppression of de-novo IAA biosynthesis.

George (1993) stated that 2,4-D is primarily used for callus induction instead of NAA and IBA which are generally required for morphogenesis. Furthermore, NAA and IBA are favorable auxins for shoot culture or root formation. Shu et al. (2005) have also demonstrated that 2,4-D was more efficient than NAA for callus formation of stem, petiole and leaf explants of *Dioscorea zingiberensis*. Meanwhile, Guo and Zhang (2005) reported that 2,4-D plays an important role in inducing the formation of embryogenic calli and somatic embryogenesis in ginger. They reported that cell division in the epidermal cells was triggered by the use of 2,4-D, and developed further into somatic embryos.

The result in the present study shows that 8 mg/L of 2,4-D was among the concentration which produced the

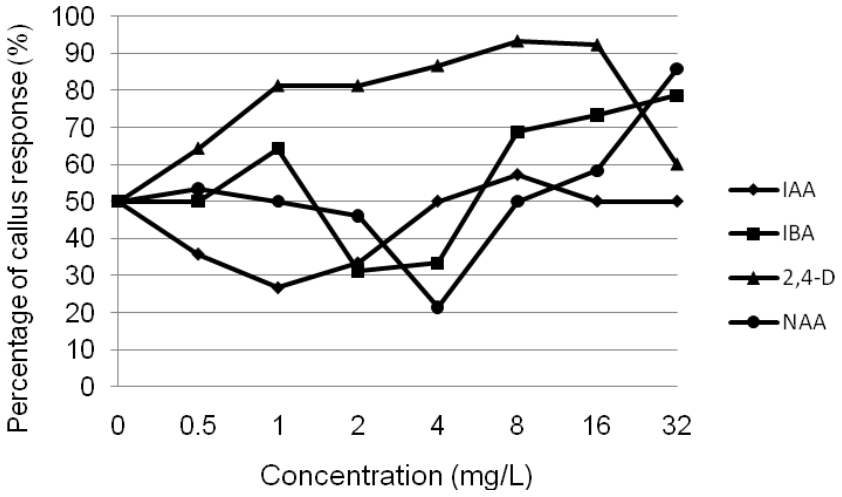

Figure 1. The effect of auxin type and concentration on the percentage of callus response from mangosteen, *G. mangostana* seed explants after 8 weeks of culture.

Table 2. The effect of explant types, auxin type and concentration on callus fresh weight, the percentage of callus response, callus score, and percentage of contamination in mangosteen, *G. mangostana* after 8 weeks of culture.

Source of variance	Mean			
	FW (g)	PC	CS	POC
Explant types				
Coated	169.53^{a}	72.22^{a}	2.38^{a}	25^{a}
Uncoated	59.98^{b}	44.23^{b}	1.66^{b}	9.72^{b}
Auxin types				
IAA	99.35	44.16^{c}	1.70^{b}	16.67
IBA	100.38	55.83^{b}	1.92^{b}	16.67
2,4-D	116.67	75.83^{a}	2.47^{a}	16.67
NAA	122.75	51.72^{bc}	1.84^{b}	19.44
Concentration (mg/L)				
0	1383.1	50^{b}	1.81^{b}	11.11
0.5	1163.2	50.87^{b}	1.91^{ab}	20.83
1	895.4	55.73^{ab}	1.95^{ab}	15.28
2	1077	48.33^{b}	1.81^{b}	16.67
4	1392.2	48.33^{b}	1.93^{ab}	16.67
8	1116.8	67.21^{a}	2.21^{a}	15.28
16	795.7	68.51^{a}	2.14^{ab}	25
32	900.4	67.79 ^{a}	2.15 ^{ab}	18.06

FW= Fresh weight (mg); PC= percentage of callus response; CS = Callus score; POC= percentage of contamination. Means with the same letters of each factor in each column and of each parameter are not significantly different at α = 0.05.

highest percentage of explants producing callus (93.3%) which was not significantly different with the percentage of callus obtained on 0.5, 1, 2, 4, 16 and 32 mg/L 2,4-D; 1, 8, 16 and 32 mg/L IBA and 16 mg/L NAA (Data not shown). However, 2,4-D at 8 mg/l was considered the most favorable treatment. Explant type significantly affected the percentage of explants producing callus. The coated explants produced higher percentage of callus response (72.22%), the higher callus score (2.38) and fresh weight of callus (169.53 mg) compared to uncoated explants (44.23 %), (1.66), and (59.98 mg) respectively, as shown in Table 2. Uncoated seed explants have cut

ends in all sides. Callus is often induced in or upon contact of the wounded part of the explants with the media. Callus may be formed on all those wounding sides. However, the formation of callus was inhibited by the browning of explants which is caused by the oxidation of phenolic compounds released by the wounded cells. Tang and Newton (2004) demonstrated that tissue browning is associated with the accumulation of polyphenol oxidase and decrease of putrescine, spermidine, and spermine in browning calli, which inhibited callus growth in Virginia pine. However, explants wounding was kept to a minimal as coated seed explants only had two cut ends, and was placed in contact with media. The wounding side absorbs nutrient and PGRs from the media and induce callus on the coated layer. Moreover, the browning percentage on coated explants was lower (78.8%) when compared to uncoated explants (91.1%). This promoted the higher percentage of callus response obtained on coated explants compared to the uncoated ones.

To survive and grow properly, *in vitro* plant cultures need to be largely free from pest, fungi and bacterial infections. The result in the present study showed that explant type affected the percentage of contamination (Table 2). The coated explants induced higher percentage of contamination (25 %) compared to the uncoated explants (9.7%) as shown in Table 2.

Both coated and uncoated explants were surface sterilized, however, the outer layer on some coated explants may contain fungus or bacterial spores which were not present on the uncoated explants. The coats were removed from the uncoated explants after surface sterilization. The coats may have caused the higher percentage of contamination on the coated explants compared to the uncoated explants. This indicated that uncoated explants were better in preventing explants contamination.

Texture and color of callus

Both coated and uncoated seed segments that were cultured on LS medium supplemented with IAA, IBA or NAA resulted in the formation of spongy, whitish and yellowish calli compared to the calli produced from 2,4-D treatments, whereby the calli appeared as yellowish compact nodules. The compact calli texture from medium containing 8 mg/L 2,4-D developed into friable texture after monthly subculture onto fresh medium. Chai et al. (2010) demonstrated the initiation of calli of *Zoysia matrella* after 3 to 5 days inoculation within MS medium supplemented with 2 mg/L 2,4-D, the calli obtained were white, fast-growing and loose in structure, and mostly non-embryogenic. However, after 2 months of subculture, compact, nodular, yellowish to yellow structure (embryogenic callus) was observed on the surface of some induced calli.

A compact-yellowish tissue was observed on media supplemented with 8 mg/L 2,4-D, indicating the initial stages of calli formation. However, these calli did not progress into somatic embryo stages. In the culture of most plant species, 2,4-D is relatively more effective for the induction of embryogenic calli compared to other PGRs. However, in this study, 2,4-D alone did not induce calli to form embryogenic stages. This indicates the need for a combined treatment of 2,4-D and other PGRs which could stimulate callus to form somatic embryos.

Based on the aforementioned results, 2,4-D is the most favorable auxin for the induction of calli from mangosteen seed explants as shown on all parameters observed. The concentration of 8 mg/L 2,4-D was the most favorable for high percentage of callus response from explants. Uncoated explants were chosen as the most favorable explant with the lowest percentage of browning and contamination, compact texture and yellowish color of callus.

The effect of 2,4 D and cytokinins on callus induction

Different media of MS and LS supplemented with different types and concentrations of cytokinin (0 and 0,1 mg/l BAP; 0, 0.1, 1, 2, 3, 4 and 5 mg/l TDZ and 0, 0.1 mg/l Kinetin) either alone or in combination with different concentrations of 2,4-D (0, 8, 16, 32 and 64 mg/l) significantly affected the percentage of callus formation and percentage of callus browning as shown in Table 3. The percentage of explants forming callus on MS medium containing 8 mg/L 2,4-D + 0.1 mg/L TDZ (86.67%) was among the highest as those on MS medium containing 8 mg/L 2,4-D + 0.1 mg/L BAP (80%) and LS medium containing 8 mg/L 2,4-D + 0.1 mg/L BAP (86.67%). These treatments also produced among the lowest percentage of calli browning (66.67, 53.53 and 66.67%, respectively). Auxin 2,4-D at 8 mg/L with BAP or TDZ showed favorable embryogenic calli responses. George (1993) reported that the combination of cytokinins and auxins stimulates cell division and cell morphogenesis in culture. BAP is among the most commonly used cytokinin compared to TDZ. Narayanaswamy (1994) reported that BAP actively interacts with 2,4 D or IAA for the initiation and maintenance of calli.

In this experiment, calli textures and color formed on the media were compact and remained greenish in color. The calli also did not proceed to embryogenic stage after 8 weeks of culture, as similarly observed in the previous study.

Narayanaswamy (1994) stated that a medium lacking certain nutrients may lead to the stimulation of friable calli formation. Some organic nutrients and amino acids may be needed for calli to develop into embryogenic stages. Frequent subcultures of calli may cause calli to become friable (Razdan, 2003).

Table 3. The effect of 2,4-D and cytokinins on the percentage of callus response, percentage of callus browning from mangosteen seed segments that were cultured on MS and LS medium for 8 weeks.

Media types	2,4-D	Cytokinin (mg/L)			Percentage of callus response	Percentage of callus browning
	(mg/L)	BAP	TDZ	Kinetin		
MS	0	0	0	0	35.71^{efghij}	92.86^{abcd}
	8	0.1	0	0	80.00^{abc}	53.53 ^{e}
	16	0.1	0	0	78.57^{abcd}	64.29^{cde}
	32	0.1	0	0	73.33^{abcde}	86.67^{abcd}
	64	0.1	0	0	26.67^{hij}	93.33^{abcd}
	8	0	0.1	0	86.67^{ab}	66.67^{bcde}
	16	0	0.1	0	78.57^{abcd}	79.07^{abcde}
	32	0	0.1	0	40.00^{defghij}	93.33^{abcd}
	64	0	0.1	0	7.14 ^{j}	93.50^{abcd}
	0	0	1	0.1	64.29^{abcdefgh}	93.57^{abcd}
	0	0	2	0.1	33.33^{fghij}	100^{a}
	0	0	3	0.1	60^{abcdefgh}	93.33^{abcd}
	0	0	4	0.1	53.33^{bcdefghi}	86.67^{abcd}
	0	0	5	0.1	92.86^{a}	93.86^{abc}
LS	0	0	0	0	30.77^{ghij}	86.92^{abcd}
	8	0.1	0	0	86.67^{ab}	66.67^{bcde}
	16	0.1	0	0	66.67^{abcdefg}	86.67^{abcd}
	32	0.1	0	0	42.86^{cdefghij}	94.14^{abc}
	64	0.1	0	0	30.77^{ghij}	87.54^{abcd}
	8	0	0.1	0	66.67^{abcdefg}	63.33^{de}
	16	0	0.1	0	71.43^{abcdef}	65.79^{bcde}
	32	0	0.1	0	28.57^{ghij}	81.79^{abcde}
	64	0	0.1	0	14.29^{ij}	100^{a}
	0	0	1	0.1	20.0^{ij}	95.00^{ab}
	0	0	2	0.1	42.86^{cdefghij}	92.86^{abcd}
	0	0	3	0.1	50.0^{bcdefghi}	68.07^{bcde}
	0	0	4	0.1	50.0^{bcdefghi}	100^{a}
	0	0	5	0.1	41.67^{cdefghij}	4.67^{f}

Means with the same letters of each parameter are not significantly different at α≤0.05.

The effect of glutamine on embryogenic callus induction

Glutamine concentrations significantly affected callus scores after 4 months of culture as shown in Figure 2. The concentration of 500 mg/L glutamine induced moderate callus scores which were not significantly different from 300 mg/L glutamine and 0 mg/L glutamine. Therefore, glutamine in the media does not affect callus growth. This result is in agreement with the fresh weight of callus obtained. Glutamine concentration does not affect callus fresh weight (Data not shown).

Although glutamine did not increase calli growth, the texture and color of calli was improved. Calli was more friable and yellow after six subcultures on media supplemented with glutamine, compared to calli produced from cultures without glutamine treatment which is brownish in color. The role of glutamine in developing somatic embryos than cell growth was observed by Higashi et al. (1996) which stated glutamine inhibit cell growth in Tobacco and having effects on the development of somatic embryos rather than on the total number of somatic embryos in Carrot. The supply of some amino acids include glutamine in the medium results in the increase of nitrate reductase activity in the cells and the increased nitrate reductase activity makes it possible to utilize nitrate, and results in the formation of some somatic embryos. This study showed that the highest concentration of glutamine applied gave the highest formation of friable calli.

The friable calli obtained in this study showed the onset characteristic of embryogenic calli. Embryogenic potential

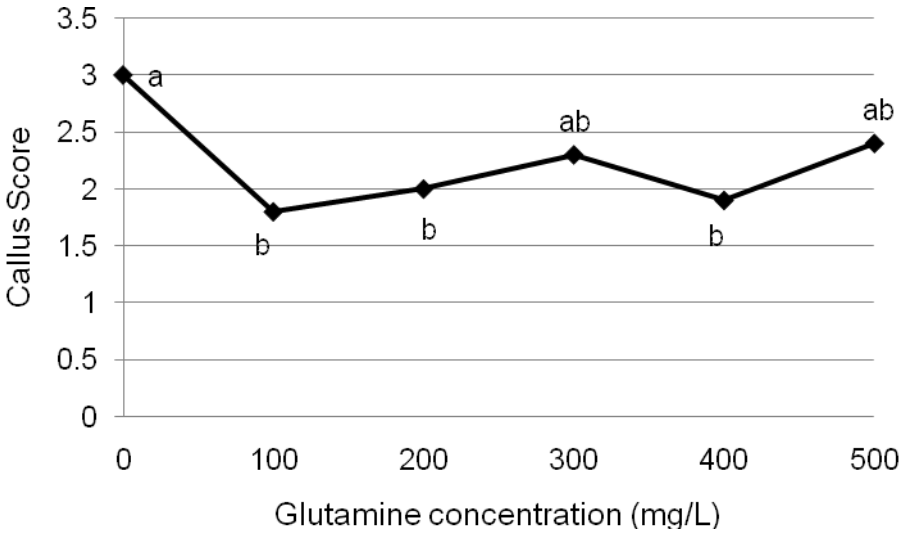

Figure 2. The effect of increasing glutamine concentrations on callus scores from mangosteen uncoated seed explants after 4 months of culture.

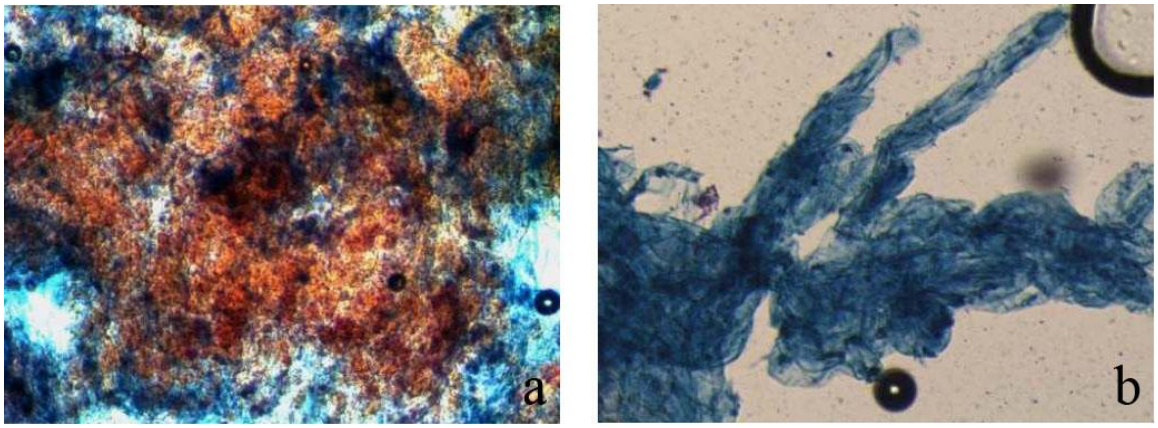

Figure 3. The color of mangosteen calli after detection by double staining method. A large portion of the embryogenic calli stained red (a) and the non-embryogenic calli stained blue (b).

in the cultures were detected by the double staining method. Jain and Gupta (2005) used the double staining method using acetocarmine and Evan's blue dye for distinguishing embryogenic and non-embryogenic cells. Embryogenic cells have large nuclei and dense cytoplasm. These nuclei stain an intense bright red with acetocarmine. Strands in the cytoplasm also show an affinity for acetocarmine and stain bright red. Otherwise, the nuclei of non embryogenic cells are very small. The acetocarmine-stained red material is difficult to locate and whole cells stain blue with Evan's blue. Evan's blue also determines the viability of cells. Less viable cells are vacuolated with small nuclei that permit Evan's blue dye to enter. In the present study, embryogenic cells were characterized by the large portion of bright red stained nucleus (Figure 3a). Non-embryogenic calli cells stained blue (Figure 3b). Microscopic observations showed that embryogenic calli were present on medium with or without glutamine as shown by the red stained areas. The study shows that the addition of glutamine in half strength MS medium containing 8 mg/L 2,4-D and 0.1 mg/L BAP induced the formation of embryogenic calli. Observation of calli under microscope showed that 500 mg/L glutamine produced the largest red area portion (+++++) compared to the other glutamine concentrations (++, +++, ++++). Based on that reason, 500 mg/L glutamine concentration was chosen as the most favorable treatment for inducing the embryogenic calli. Report by Rosseleena and Normah (2007) in the production of mangosteen globular calli using media supplemented with glutamine showed that 400 mg/L glutamine induced embryogenic calli from mangosteen

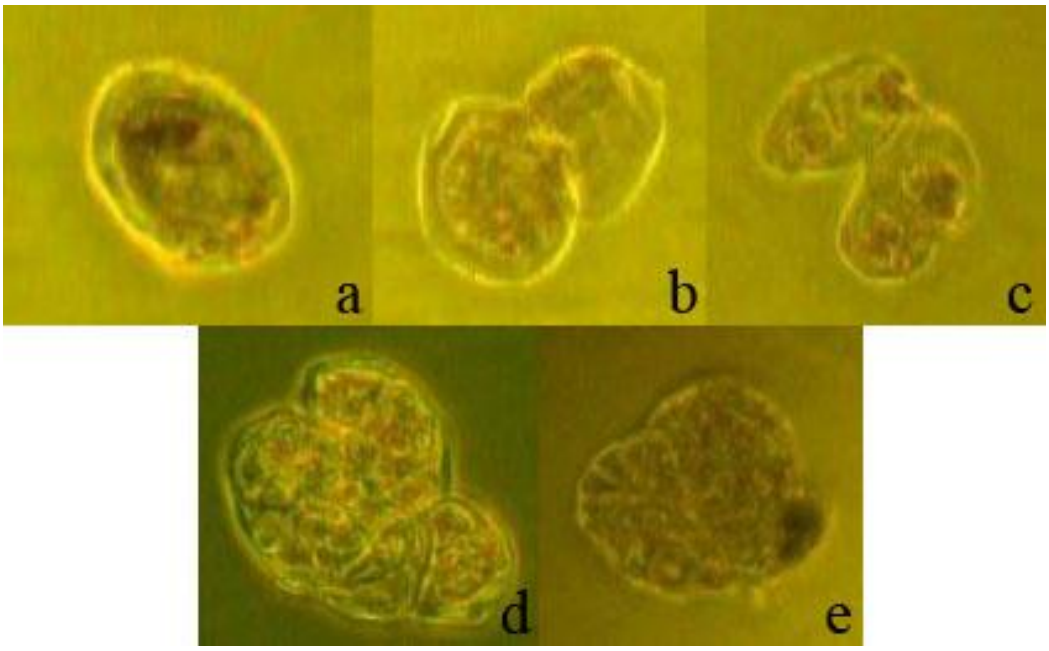

Figure 4. Development of mangosteen single cell calli to embryogenic stages on medium supplemented with 1, 3 and 9 mg/L concentrations of BAP. (a) Single cell, (b) Doubled cell, (c) Tetrad cell, (d) Cell clusters, (e) Heart stage. 10× magnification.

seed.

Development of embryogenic stages in suspension cultures

Cell multiplication

2,4-D was used as treatment to induce cell multiplication. Cell cultures were subcultured every month for 10 months. However, cells on half strength MS medium supplemented with 2, 4 and 8 mg/L 2,4-D (D2, D3, D4 treatment) were contaminated by bacteria and fungi, except for those cultured on half strength MS0 medium (D1). These surviving cell cultures were then subcultured into fresh medium and cell morphology was observed under an inverted microscope. After the first week in culture, cells were counted by using a haemacytometer. The cells showed instability in number due to the low cell density. Because of slow growth of cells, growth rate was not determined.

The cells cultured in half strength MS0 liquid medium showed a wide range of differences in cell morphology. Single cells were elongated. Moreover, empty cell without dense cytoplasm was also observed. After 10 months of culture, cells divide into two, three, four and more times. This shows that the cells were able to divide and proliferate even though cultured in MS medium without auxin. Endogenous 2,4-D auxin within the calli cultures could have stimulated cell division. Moreover, the reduction of nutrients stimulated cell division and proliferation under stress condition as reported by Narayanaswamy (1994). The cells continued to proliferate without any observed embryogenic formation. This condition was not in agreement with Nomura and Komamine (2005) where stimulated somatic embryos when embryogenic cell cultures were transferred from a medium containing auxin to an auxin-free medium. In another report by Sane et al. (2006) on *Phoenix dactylifera* suspension cell cultures, the addition of 2,4-D inhibited somatic embryogenesis from embryogenic cell clusters. Based on these reports, the cytokinin for stimulating differentiation from cell clusters into embryogenic stages may be needed.

The effect of BAP on the development of embryogenic stages

Cells cultures from D1 media was transferred into half strength MS liquid medium containing 500 mg/L casein hydrolysate with different concentrations of BAP. Cultures were subcultured every month for six months and observed morphologically under an inverted microscope. Embryoid-forming cells are characterized by dense cytoplasmic contents, large starch grains and relatively large nucleus with darkly stained nucleus (Dodds and Roberts, 1982) as shown in Figure 4a.

The single cell divided into two and four then form cell clusters (Figure 4a to d) and heart stage (Figure 4e). The

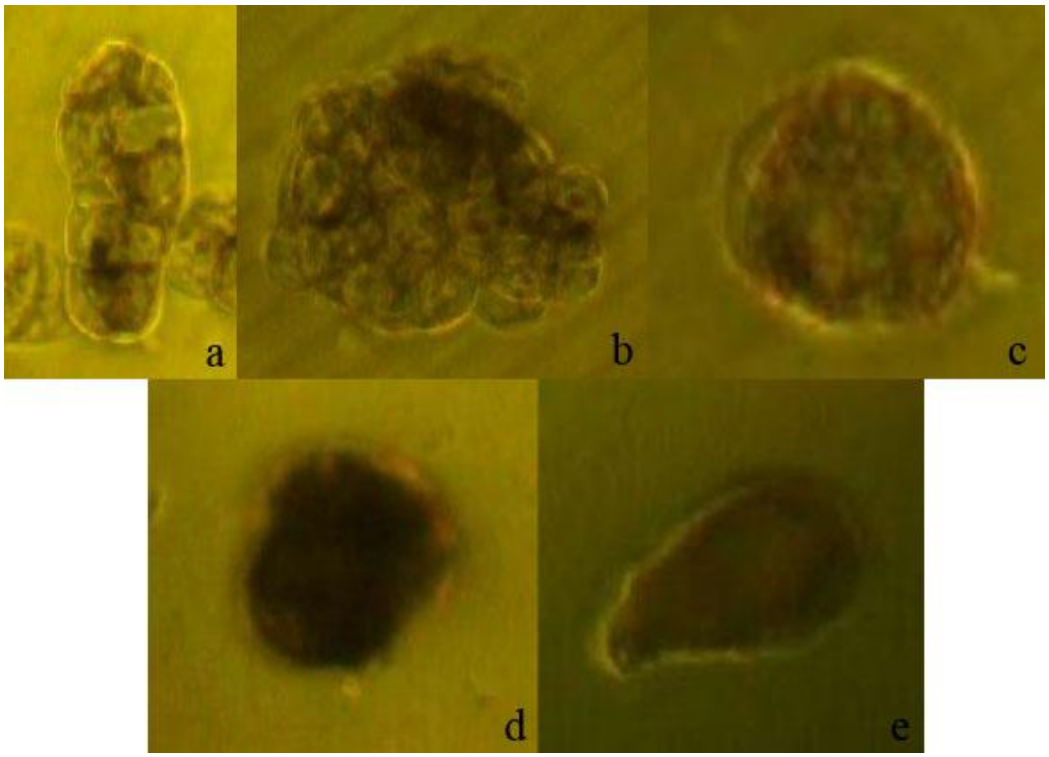

Figure 5. The development of mangosteen single cell calli to embryogenic stages of growth on MS liquid medium supplemented with various concentrations of TDZ. (a-b) Cell clusters; (c-d) Globular stage; (e) Torpedo stage. 10× magnification.

morphology of cells in half strength MS liquid media supplemented with 1, 3 or 9 mg/L BAP produced single cell and cell clusters. Furthermore, advanced embryogenic stages of the cells such as globular or heart was not frequent. After six months of culture, the heart embryogenic stage was obtained only on medium supplemented with 1 mg/L BAP. However, this result was not in agreement with Nomura and Komamine (2005) which stated that BAP could not develop embryogenic cells into somatic embryos in carrot suspension cell cultures. In contrast, Te-chato et al. (2008) reported the ineffectiveness of BAP to enhance cell growth in oil palm suspension cultures. Combined treatment of 2 mg/L 2,4-D and 1 mg/L BAP also produced the growth of suspension cell cultures in *Pinus pinaster* (Azevedo et al., 2008). However, the transition of these cultures to embryogenic formations was not reported.

The effect of TDZ on the development of embryogenic stages

Cell cultures on half strength MS liquid medium containing 500 mg/L glutamine and supplemented with 0.1 mg/L TDZ was transferred to half strength MS liquid medium containing 500 mg/L casein hydrolysate and supplemented with different concentrations of TDZ (1, 3 and 9 mg/L). The cultures were subcultured every month for five months, and observed morphologically under an inverted microscope. After five months in culture, the cell clusters developed (Figure 5a to b) into globular (Figure 5c to d) and torpedo (Figure 5e) stages in half strength MS liquid media supplemented with 1, 3 and 9 mg/L. This is a new development for mangosteen, since there have been no successful reports on the development of somatic embryogenesis from cell suspension cultures before this.

In this study TDZ induced the transition of single suspension cells into more advanced embryogenic stages (torpedo). The use of TDZ for initiating and developing embryogenic stages from cell suspension cultures has been reported in *Colocasia esculenta* var. *esculenta* in medim containing 2,4-D. These calli were transferred onto solid media supplemented with 1.0 mg/L TDZ, 0.5 mg/L 2,4- D and 800 mg/L glutamine before it was transferred to liquid medium containing the same components but with reduced glutamine (100 mg/L) (Deo et al., 2010).

Conclusion

The uncoated seed segments cultured on medium supplemented with 8 mg/L 2,4-D is the favorable treat

treatment for inducing callus. The MS medium supplemented with 0.1 mg/L BAP + 8 mg/L 2,4-D was among the most favorable treatments for the induction of compact calli in texture and greenish or yellow in color. This treatment produced among the highest percentage of explants forming callus (80%) and the lowest percentage of calli browning (53.53%). The result in the third study showed that addition of glutamine into half strength MS media + 8 mg/L 2,4-D + 0.1 mg/L BAP did not increase the growth of calli but the texture and color of callus was improved. Calli produced on media with glutamine were more friable and yellow compared to the calli which were produced from non-glutamine treatment. The concentration of 500 mg/L glutamine was the most favorable treatment for inducing the embryogenic calli as shown by the largest red stained areas (+++++). Both BAP and TDZ stimulated cell growth into advanced embryogenic stages. However, heart stages were observed only on medium supplemented with 1 mg/L BAP after six months of culture. Addition of TDZ in different concentrations such as 1, 3 and 9 mg/L into medium induced the transition of single cell calli to more advanced embryogenic stages (torpedo) after five months of culture. This result is an important discovery as *in vitro* propagation of mangosteen on suspension cell cultures, have not been successful in developing somatic embryogenesis in previous reports.

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