ORIGINAL ARTICLE

Plantlet Formation from Leaf-Derived Embryogenic Callus of Mangosteen

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Abstract

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Embryogenic callus derived from culture purple leaves of mangosteen was multiplied and maintained in Murashige and Skoog (MS) medium supplemented with 500mg/l polyvinylpyrolidone (PVP), benzyladenine (BA) and thidiazuron (TDZ) at the same concentration of 0.5mg/l. Transfer the callus to Woody Plant Medium (WPM) in the presence of BA alone at concentration 0.1mg/l promoted induction of leaf primodia and elongation of the shoot after 2-3 times of subculture. Elongated shoots were excised and successfully induced root by making wound at the basal part of the shoot subsequent to dipping in 1,000 mg/l indole butyric acid (IBA) in darkness for 15 minutes. The shoots were then transferred to woody plant medium (WPM) supplemented with 0.25mg/l BA, 5.6mg/l phloroglucinol (PG) and 0.25% activated charcoal. The cultures were firstly kept in the dark for 2 weeks and transferred to illuminate condition for further 2-4 weeks.

Key words : plantlet formation, embryogenic callus, mangosteen

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การชักนำพืชต้นใหม่จากเอ็มบริโอเจนิคแคลลัส ซึ่งชักนำจากใบมังคุด

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การเพิ่มปริมาณและการดูแลรักษาเอ็มบริโอเจนิคแคลลัสที่ชักนำจากใบสีม่วงของมังคุดเป็นไปได้ดีในอาหารสูตร MS เติมโพลีไวนิลไพโรริโดนเข้มข้น (PVP) 500 มิลลิกรัมต่อลิตร เบนซิลอะดินีน (BA) และ ไธไดอะซูรอน (TDZ) ความเข้มข้นเท่ากัน 0.5 มิลลิกรัมต่อลิตร เมื่อย้ายแคลลัสไปเลี้ยงในอาหารสูตร WPM เติม BA เพียงลำพัง ความเข้มข้น 0.1 มิลลิกรัมต่อลิตร ส่งเสริมการสร้างใบคู่แรกและการยืดยาวของยอดหลังการย้ายเลี้ยง 2-3 ครั้ง การชักนำรากจากยอดให้ผลสำเร็จสูงโดยการทำแผลที่โคนของยอดแล้วจุ่มแช่ในสารละลายกรดอินโดลบิวทีริค (IBA) เข้มข้น 1,000 มิลลิกรัมต่อลิตรในที่มืดเป็นเวลา 15 นาที ย้ายยอดที่เตรียมได้ไปเลี้ยงในสูตรอาหารเลี้ยงไม้ยืนต้น (WPM) เติม BA เข้มข้น 0.25 มิลลิกรัมต่อลิตร โฟลโรกลูซิโนล (PG) เข้มข้น 5.6 มิลลิกรัมต่อลิตร และผงถ่านเข้มข้น 0.25 เปอร์เซ็นต์ ในช่วงแรกเลี้ยงในที่มืดเป็นเวลา 2 สัปดาห์ แล้วจึงย้ายไปเลี้ยงในที่ให้แสงต่ออีกเป็นเวลา 2-4 สัปดาห์

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There are few reports on shoot organogenesis of mangosteen.[3,4,8,9,10] Those reports found some problems, especially root induction from *in vitro* shoot that hampered mass scale propagation of the fruit tree. However, by manipulation culture medium and phytohormones together with pre-treatment the shoot before rooting, it has been reported to enhance root induction with 100% success.[9] Somatic embryogenesis is an alternate plant regeneration procedure. The procedure may advantage over shoot organogenesis due to higher rate of multiplication. For woody plant species, there are few reports on somatic embryogenesis using thidiazuron (TDZ).[1,2,5,6,7] The most common explants for plantlet regeneration through somatic embryogenesis are cotyledon from both mature and immature seeds and leaf tissue from *in vitro* culture.[7] Somatic embryogenesis and rhizogenesis is, at present, one of its major goal. Earlier results obtained in our laboratory showed the potential of mangosteen to use as a model system for studying *in vitro* morphogenesis in fruit trees.[11,12] The development of culture systems for controlled induction of direct and indirect embryogenesis and rhizogenesis was the first step to improve mangosteen using biotechnology (including genetic transformation). From those results induced purple leaf explants are the most desirable explants.

In this paper we report the processes of plantlet regeneration from embryogenic callus derived from purple leaves of mangosteen.

Materials and Methods

Embryogenic callus induction:

Seeds of mangosteen were aseptically induced multiple shoots in modified Murashige and Skoog (MS) medium. Seed with cluster of shoots were subsequently transferred to liquid 1/2MS medium. The detailed procedures were already described by Te-chato *et al.*[8,10] Purple and green leaves were dissected and transferred to callus induction medium. The medium was MS medium supplemented with 500mg/l polyvinylpyrrolidone (PVP), 0.5mg/l benzyladenine (BA) and 0.5mg/l TDZ. The medium was solidified by 0.2% Gelrite or Gellan gum. The cultures were maintained at 28°C under 2,000 lux illumination for 16 hour photoperiod and subculture was carried out monthly interval for 3 months.

Germination of somatic embryos:

Early staged somatic embryos developed on induction medium were transferred to both liquid and solid of 2 different culture media, MS and Woody

Plant Medium (WPM). Liquid culture media were supplemented with 0.06mg/l naphthaleneaceti acid (NAA) and 0.03mg/l BA. Solid culture media were modified by supplementation with 500mg/l PVP, 30g/l sucrose and BA at various concentrations as following;

1. WPM and MS with 1.0 mg/l BA
2. WPM with 0.1, 0.5, 1.0 mg/l BA

The cultures were maintained in the same way as embryogenic callus induction. Subculture was carried out monthly interval for 3 months. At the end of culture period, a number of shoots at various sizes were recorded and compare statistically among those culture media and concentrations of BA.

Root induction:

Elongated shoots with length more than 2 centimeters were excised and made wounds at the basal cut end of the shoots. The shoots were then dipped in the solution of 1,000mg/l IBA (sterilized by filtering through millipore membrane with pore size 0.45μ) in the dark for 15 minutes. The shoots were finally transferred to WPM medium modified by supplementation with 0.25mg/l BA, 0.25% activated charcoal and phloroglucinol (PG). PG was added to the medium at various concentrations. Initially, the cultures were miantained under darkness for 2 weeks to induce root primordia. To promote elongation of the root the cultures were subsequently transferred to illuminate with 2,000 lux, 16 hour photoperiod at 26°C for further 2 to 4 weeks. At the end of culture period, percent root formation, number and length of root were scored and statistically compared.

Results and Discussion

Somatic embryos induced in induction medium ceased to undergo further stage of development. A new somatic embryo, so called additive embryos or additional embryos, was observed. After removing TDZ from culture media, somatic embryos could develop beyond globular stage. Leaf primodia appeared after a month of culture (Figire 1). In liquid culture media, MS was proved to be better for leaf primordia induction whereas WPM promoted a severe browning of somatic embryos (Table 1).

Adversely effect was obtained when cultured onto solid medium. WPM medium was better than MS medium. WPM medium supplemented with 1 mg/l BA provided greenish color of somatic embryos with leaf primodia. Even percent shoot bud formation was not significantly observed but average number of shoots per callus was far significant difference (Table 2). BA at high concentration (1 mg/l) inhibited elongation of the shoots. The shoots obtained in culture medium with 1mg/l BA was clustered. Total number of shoots was inferior to BA at concentration 0.5 and 0.1 mg/l. BA at low concentration of 0.1 mg/l provided the highest elongated shoot (Figure 2), followed by concentra-

Table 1. Effect of liquid culture media on shoot development from embryogenic callus of mangosteen.

Culture media	Number of shoot at various sizes			Browning of somatic embryos
	0-0.5 cm	0.6-1.0 cm	>1.0 cm	
WPM	3.13b	1.40b	0	++++
MS	6.80a	2.17a	0.67	+
F-test	*	*	ns	
cv(%)	26.30	18.31	44.95	

* : Significant difference at P=0.05
ns: Not significant
Mean not sharing letter in common within column is significant difference at P=0.05 by DMRT
+: Slightly occured,
++++: Severely occured

Table 2. Effect of two different solid culture media with 1 mg/l BA on shoot bud formation from embryogenic callus of mangosteen.

Culture medium	Shoot bud formation (%)	Number of shoots per callus
MS	33.33	1.67b
WPM	58.33	4.00a
F-test	ns	*
cv(%)	62.98	28.82

*** : Significant difference at P=0.05**
ns : Not significant
Mean not sharing letter in common wihtin column is significant difference at P=0.05 by DMRT

Table 3. Effect of concentrations of BA on shoot bud formation in WPM medium

concentration (mg/l)	no. of shoot at length		
	0-0.5cm	0.6-1.0cm	>1.0cm
1.0	5.90b(1-13)	0.48(0-5)	0.22(0-5)
0.5	8.39a(3-19)	0.94(0-6)	0.48(0-5)
0.1	9.27a(2-19)	0.97(0-7)	0.58(0-6)
F-test	*	ns	ns
cv(%)	17.59	70.19	87.75

*** : Significant difference at P=0.05**
ns: Not significant
The number in parenthesis showed minimum and maximum number of shoots at those length.
Mean not sharing letter in common within column is significant difference at P=0.05 by DMRT

tion 0.5 and 1.0 mg/l, respectively (Table 3). Shoots obtained from all concentrations of BA elongated very slowly due to heterogeniety of development.

100% root induction could be achieved from excised single shoots in root induction medium. PG at all concentrations could induce root primodia within 2 weeks. A 100% root primodia induction was obtained in the medium supplemented with 5.6 mg/l PG. However, percent root induction was reach to 100% in the medium supplemented with all concentrations of PG at 4 weeks of culture. Significant difference was not observed among concentrations of PG but the higher concentration tended to induce higher quality of root in terms of root number and root length (Table 4, Figure 3).

A good quality and high number of embryogenic callus induction were not obtained in the medium without TDZ. Even TDZ in both initially culture medium and subculture medium could induce embryogenic callus, but the addition of TDZ to culture medium initially promoted embryogenic callus the best. Multiplication and maintenance of the callus was regulary carried out in MS medium with 500mg/l PVP, BA and TDZ at the same concentration of 0.5mg/l. Increased efficiency of embryogenesis in TDZ-induced cultures was due to continued production of somatic embryos. Continuous exposure of the callus to TDZ is not essential for achieving maximum morphogenetic response. Remove TDZ from the medium promoted further development stage of somatic embryos. WPM or MS medium supplemented with BA alone at concentration 1.0 mg/l could induce leaf primodia subsequent to elongation of the shoot. Compare between WPM medium

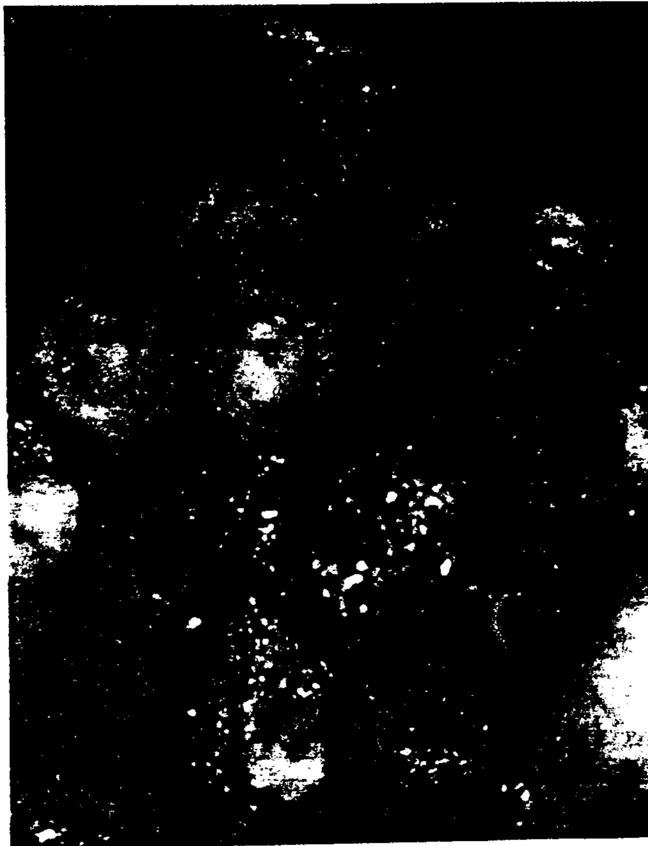

Figure 1. Leaf primodia induction in WPM medium without TDZ.

Figure 2. Elongation of the shoot in WPM medium with 0.1mg/l BA.

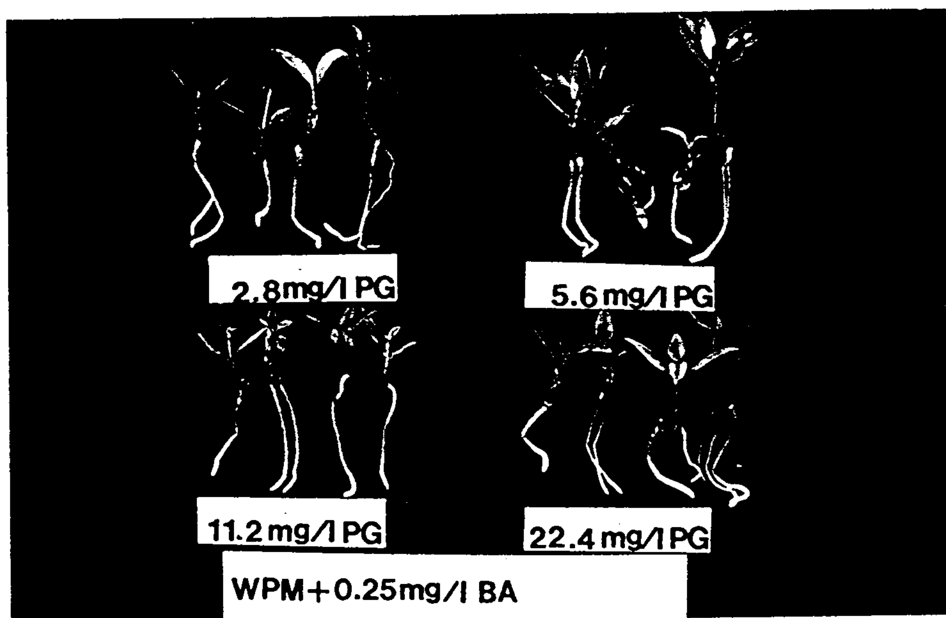

Figure 3. Root formation in root induction medium with various concentrations of PG.

Table 4. Effect of PG at various concentrations on root induction from vitro-micro-shoots of mangosteen.

concentration of PG mg/l	average root induction (%)	average root number	average root length (cm)
0	35b	0.90	2.55
2.8	100a	1.55	3.22
5.6	100a	1.55	3.09
11.2	100a	1.60	2.96
22.4	100a	1.35	3.45
F-test	*	ns	ns
cv(%)	**8.94**	**16.94**	**10.77**

*** : Significant difference at P=0.05**
ns: Not significant
Mean not sharing letter in common wihtin column is significant difference at P=0.05 by DMRT

and MS medium, WPM medium has stronger components than MS medium, especially potassium sulfate (K_2SO_4) and calcium nitrate ($Ca(NO_3)_2$) which was not present in MS medium. Those components in form of liquid medium caused easily browning of the callus due to a high absorption rate. Generally, elongation of the shoot need a low concentration of BA. BA at a high concentration was proved to induce a large number of tiny shoots or multiple shoots but inhibit elongation of the shoots. Shoots developed in shoot induction medium was heterogeneous. Competition between shoots for uptaking nutrient in the medium occured. The faster the shoot come out, the more and faster develop to be a healthy shoots. The shoots at length about 2 centemeters were successfully induced root with 100% in root induction medium. Root induction will never be obtained if the shoot was not wounded at the basal part and dipped in a high concentration of IBA solution. The roots primodia come out from wound. The darkness condi-

tion promoted the effect of IBA in inducing root primodia. Induction root in continuous illumination was inferior due to photodegradation of IBA. Naphthalene acetic acid (NAA) or indole acetic acid (IAA) containing medium was also reported to induce root with 100 %[9] but time consumed for induction root and quailty of root was inferior to PG.

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