ORIGINAL ARTICLE

Effect of gelling agents on shoot growth and multiple shoot formation of mangosteen

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Abstract

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Apomict seeds of mangosteen were cultured as a whole or half seed on MS medium supplemented with 5 mg/l 6-benzyladenine (BA). The medium was solidified with various gelling agents. After culture for 2 months, multiple shoot formation, morphological and physiological characters of the shoot were investigated. The results revealed that 1.5% agarose gave the highest seed forming shoot (98%) and number of shoots per culture seed (20.7). Wounding the seed by sectioning into half promoted higher callus formation (47-88%) in all gelling agents. Phytagel (0.17%) resulted in the highest callus formation (100%) and hyperhydric shoots (11-31%). Those shoots produced translucent, thin and brittle leaves and stems, and malformed stoma. Those leaves had the lowest content of chlorophyll a, b and total chlorophyll.

Key words : mangosteen, multiple shoot, gelling agent, hyperhydricity, chlorophyll content

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บทคัดย่อ

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ผลของวุ้นต่อการเจริญและการสร้างยอดรวมมังคุด

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ผลการเพาะเลี้ยงเมล็ดมังคุดทั้งเมล็ด หรือตัดแบ่งครึ่งบนอาหารสูตรมูราชิกและสกุด (MS) เติมเบนซิลอะดินิน (BA) 5 มก./ล. และวุ้นชนิดต่าง ๆ ตรวจสอบการสร้างยอดรวม ลักษณะทางสัณฐานและสรีรวิทยาของยอด หลังจากเพาะเลี้ยงเป็นเวลา 2 เดือน พบว่า อากาโรส 1.5% ให้อัตราการสร้างยอดรวม (98%) และจำนวนยอดรวมที่สร้างต่อเมล็ด (20.7 ยอด) สูงสุด การสร้างแผลโดยการตัดแบ่งเมล็ดเป็นสองส่วนส่งเสริมการสร้างแคลลัส (47-88%) ในอาหารเติมวุ้นทุกชนิด วุ้นไฟตาเจลส่งเสริมการสร้างแคลลัส (100%) และยอดแก้ว (11-31%) สูงสุด ยอดดังกล่าวมีใบและลำต้นใส บาง เปราะและมีรูปร่างปากใบผิดปกติด้วย ใบดังกล่าวมีปริมาณคลอโรฟิลล์เอ บี และคลอโรฟิลล์รวมต่ำที่สุด

ภาควิชาพืชศาสตร์ คณะทรัพยากรธรรมชาติ มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา 90112

It is known that gelling agents supplemented in culture medium play role in growth and development of plant cultured *in vitro*. Generally, the concentration of gelling agents have a close relation with water stress. A high concentration of gelling agent causes a high water stress leaded to the difficulty of uptaking water and elements from culture medium (Scholten and Pierik, 1998). Many authors reported the influence of gelling agent on development of embryos (Stoltz, 1971), shoot apical meristems (Romberger and Tabor, 1971), somatic embryos (Tremblay and Tremblay, 1991), protoplasts (Koda *et al*., 1988), and anther (Kohlenbach and Wernicke, 1978) in *in vitro* culture. Both type and quality of gelling agents also produce problems related with hyperhydricity and necrosis of the tissue. Difference in quality of gelling agents from brand-to-brand and batch-to-batch differences may occur.

It is not known whether gelling agents which have a good performance with all plant species can be selected. To evaluate the quality of gelling agents seems to take a long time. In this report, the different groups of gelling agents are selected and tested for developmental processes of shoots of mangosteen. In addition, morphological, physiological and chlorophyll assay are discussed.

Matetials and Methods

Seeds of mangosteen were prepared for culturing as described by Te-chato and Lim (1999). Culture medium used in this study was MS and MMS supplemented with 3% sucrose and 5 mg/l BA. The details of medium components were described by Te-chato and Lim (1999). The medium was solidified with four different gelling agents; phytagel, agar-agar, agarose and Difco bacto agar. The most suitable quantity or concentration of these gelling agents as recommended by the manufacturing companies was used. The seeds were cultured in a culture bottle (8 ounce) with 15 ml medium. Each bottle contained one explant and the culture bottles were incubated at 26±2ºC with a light period of 14 h (20 μmol/m^2/s).

Morphological observartion

Leaves and stem from developed shoots *in vitro* obtained from culturing seed in culture medium supplemented with various types of gelling agents were collected. Shape and size of stems and leaves were measured and compared. Moreover, their colors were identified as either light green (translucence) or dark green (opaque). In case of stomatal size and density study, epidermis of ventral portion was peeled and placed on glass

slide, mounted with distilled water and covered with cover slip. Prepared slides were observed under inverted microscope.

Chlorophyll analysis

Samples of leaves were collected from culturing seed or seed segment on culture medium supplemented with four types of agars for 2 months. One gram fresh weight of the leaf was ground in 80% acetone solution (40 ml). The mixture of acetone and leaf pellet was filtered using Whatman paper No.1. Filtrate was adjusted the volume to 100 ml using acetone and brought to measure optical density (OD) by spectrophotometer at wavelength 645 and 663nm. The measured values were used to calculate chlorophyll content according to Withan *et al*. (1996) (quoted by Sujaree, 1997) from the following equations:

$$Ch_a = [127(D_{663}) - 2.69(D_{645})] \times \frac{V}{1000 \times W}$$

$$Ch_b = [22.9(D_{645}) - 4.68(D_{663})] \times \frac{V}{1000 \times W}$$

$$\text{Total Ch} = [20.2(D_{645}) + 8.02(D_{663})] \times \frac{V}{1000 \times W}$$

D645 and D663 are optical density at wavelength 645 and 663 nm
V is the final volume of extracted chlorophyll
W is the fresh weight of the leaf

1. Effect of wounding and gelling agents on callus and multiple shoot formation

Excised apomictic seeds were surface sterilized using the method described by Te-chato and Lim (1999). The seeds were cultured as a whole (without wounding) or segment (wounding) on MS medium supplemented with 30 g/l sucrose, 5 mg/l BA and four different types of agar; agar-agar, Difco bacto agar, agarose and phytagel at concentration of 7.5, 7.5, 15 and 1.7 g/l, respectively. The cultures were carried out in 4 ounce bottle under 10 µmol/m^2/sec, 14 h photoperiod at 28±2°C. After culture for a month a number and percentage of seed or seed segment forming callus or multiple shoot were scored and compared among those types of agar used. Moreover, abnormality of the shoots, leaves and stems in terms of hyperhydricity was recorded.

2. Effect of gelling agents on morphological and physiological characteristics of the leaves and stems

The leaves and stems of mangosteen raised on four different types of agar containing MS medium supplemented with 5 mg/l BA (as obtained from Expt. I) for two months were compared. Morphological characteristics were identified based on their shape, size and color of stem and leaf. In case of physiological characteristic, stomatal density and size of normal and hyperhydric leaf epidermis (1 mm^2) were compared. Moreover, chlorophyll content in the leaf obtained from seed-derived shoots in four types of agar containing the medium was measured and compared.

Results

1. Effect of wounding and gelling agent on callus and multiple shoot formation

Wounding played a major role on both number of shoot development and callus formation. The number of shoots slightly decreased when the seeds were cultured as segment. Contrary result was obtained in case of callus formation. Segmented seed or wounding showed a higher rate of callus formation in all types of agar especially phytagel (Table 1). In the case of abnormal shoots in term of hyperhydricity, severe syndrome was observed mostly in phytagel-solidified culture medium. Segmented seeds gave callus far greater than that of whole seed (Table 1).

In general, multiplication of magosteen plantlets is carried out using whole seed culture. BA alone containing culture medium gave better result than KN and TDZ. BA in combination with NAA was reported to promote a large number of shoot formation from sectioned seed (Normah *et al*., 1995). Many attempts had been made in this study but no shoot produced from all seed segments. Addition of NAA to the medium caused severe browning of seed explant and culture medium as

Table 1. Effect of gelling agent and wounding on callus and multiple shoot formation from cultured seed in MS medium supplemented with 5 mg/l BA for 2 months.

Gelling agent	Wounding	Seed forming callus (±SD)	Seed forming shoot (±SD)	No. of shoot/ cultured seed or seed segment (±SD)	Hyperhydric shoot development (%)
Agar-agar	No	33.70±9.96	77.72±6.25	2.01±0.56	0
	Yes	47.06±4.05	60.40±3.21	6.44±1.76	0
Agarose	No	61.31±4.15	98.04±4.40	20.73±4.36	0
	Yes	77.78±11.78	80.67±11.0	10.07±1.86	4.54±0.77
Bacto-agar	No	14.81±5.42	77.91±5.43	2.79±0.71	0
	Yes	70.73±5.64	74.01±6.59	4.81±0.19	0
Phytagel	No	100.00±0.00	97.03±4.40	19.22±1.80	11.11+1.54
	Yes	87.96±10.52	92.12±4.51	16.05±3.96	31.18+1.08

SD = Standard deviation

Table 2. Morphological characteristics of normal and hyperhydric leaf and stem obtained from culturing seed on MS medium supplemented with 5 mg/l BA for 2 months.

Leaf type	Leaf shape	Color	Width (cm) (±SD)	Length (cm) (±SD)	Diameter (mm(±SD)
Leaf					
- Normal	Ovate	Dark green	0.52±0.03	1.02±0.28	-
- Hyperhydric	Lanceolate	Light green	0.38±0.01	3.88±0.35	-
Stem					
- Normal	Opaque	-	-	-	4.4±0.03
- Hyperhydric	Translucent	-	-	-	2.6±0.01

SD = Standard deviation

well. Callus obtained from seed segment was loose, spongy-like tissue and brown in color. This callus could not be multiplied and regenerated to plantlet. This evident was also observed in culturing segmented leaf or wounding of the leaf by notching (Te-chato, 1997). In the case of gelling agents, 0.6-0.8% agar concentration is suitable for *in vitro* proliferation of pear (Kadoka and Niimi, 2003) and carnation (Yadav *et al*., 2003). Our study indicated that agarose induces more shoot and shoot growth than other gelling agents. However, few authors have compared the effects of gelling agents on shoot proliferation of fruit trees. These results suggested the use of agarose as gelling agent to increase a large number of shoots without hyperhydricity.

2. Effect of gelling agent on morphological and physiological characteristics of the leaves and stems

Types of agar play role on quality of the shoot produced in culture medium. Morphology of the leaves and stems changed in shape (width x length) and color as shown in Table 2 and Figure 1. Most of abnormal morphological characters obtained from the shoots in phytagel-solidified medium. The leaves developed in this medium had light green color and lanceolate shape, whereas normal leaves developed in agarose-solidified medium were a dark green and ovate shape (Table 2). In case of stems, they were thin and translucent with light green color in phytagel-solidified medium. Physiological characters of the shoots were focused

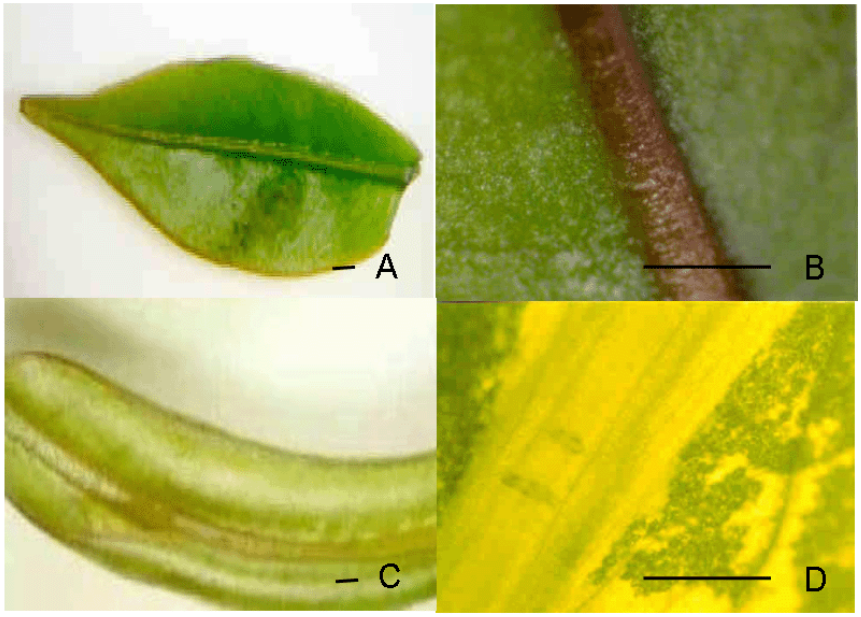

Figure 1. Morphological characteristic of normal (A, B) and hyperhydric leaves (C, D) obtained from seed-derived shoots on MS supplemented with 5 mg/l BA (bar = 0.5 mm).

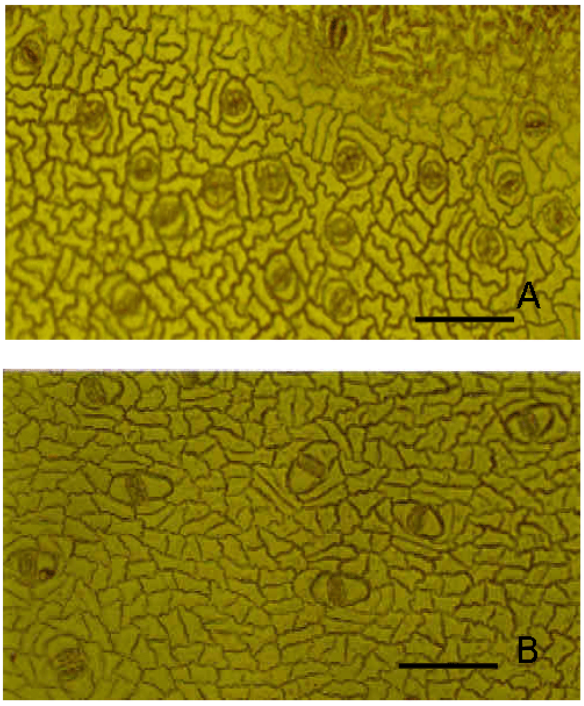

Figure 2. Characteristics and distribution of stoma in epidermal layer of normal (A) and hyperhydric leaves (B) (Bar = 60µm).

Table 3. Cholorophyll content in leaf sample of mangosteen shoot derived from culturing seeds on MS medium supplemented with 5 mg/l BA and four gelling agent for 2 months.

Gelling agent	Chlorophyll content (mg/g fr wt)		
	Chlorophyll a	Chlorophyll b	Total chlorophyll
Agar-agar	1.000b	0.530b	1.530b
Agarose	0.813c	0.460c	1.273c
Bacto-agar	1.094a	0.580a	1.674a
Phytagel	0.751d	0.402d	1.153d
F-test	**	**	**
C.V. (%)	0.15	0.31	0.23

**** Significant at P<0.01**
Means not sharing a common letter within a column are significantly different by DMRT.

Table 4. Stomatal size and density of normal leaves obtained from agarose medium and of hyperhydric leaves from phytagel medium.

Type of leaf	Stomatal size (μm)		No. of stoma/mm² (±SD)
	Width (±SD)	Length (±SD)	
Normal	40.00±5.01	47.50±2.50	18.00±2.78
Hyperhydric	34.17±5.83	63.30±11.7	10.00±3.54

on chlorophyll content with related to morphological characters. Hyperhydric shoots induced in phytagel-solidified medium showed the lowest content of chlorophyll content, both chlorophyll a and chlorophyll b (Table 3). Looking at stoma containing in the leaves, the number and distribution of stoma of the leaves from various gelling-solidified medium were quite different. Phytagel-solidified medium which yielded the highest hyperhydric leaves gave the lowest number and distribution of stoma in epidermal layer (Table 3, Figure 2). The shape, measured as width and length of stoma was also altered. Stoma in hyperhydric leaves had greater length than width (Table 4) leading to a lanceolate shape whereas nearly equal sizes were obtained in normal ones (Figure 2).

Hyperhydricity causes translucent, thin and brittle leaves and stems, including malformation of stoma. This phenomenon is also a serious problem during *in vitro* culture of carnation, which directly affects the production at commercial scale (Yadav *et al*., 2003). *In vitro* cultured plantlets do not survive when transferred to soil due to yellowing, swelling, glassiness and leaf curling of plantlets. These morphological changes are related to the low photosynthetic capacity of the leaves. Mujib and Pal (1995) and Olmos and Hallin (1998) also reported the difficulties of *in vitro* grown carnation plantlets during acclimatization in glass-house due to hyperhydricity leading the change in anatomical and morphological characteristics. So far, production of mangosteen plantlets *in vitro* showed no severe syndrome of hyperhydricity under standard protocol (Te-chato and Lim, 2000). Replacement of commercially agar with phytagel or gellan gum promoted a high frequency of hyperhydricity. This gelling agent was reported to produce hypehydricity in apple (Singha, 1982;

Turner and Singha, 1990) and pear (Banno *et al*., 1989; Yeo and Reed, 1995; Shibli *et al*., 1997; Kadota *et al*., 2001; Kadota and Niimi, 2003). It is evident in this present study that phytagel caused a low content of chlorophyll (a, b and total chlorophyll) far less than the other gelling agents. Beside types of gelling agents, its concentration was also reported to play an important role in hyperhydricity in carnation (Yadav *et al*., 2003).

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