การเกิด somatic embryo ในอ้อย

Somatic embryogenesis in sugarcane (*Saccharum officinarum* L.)

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บทคัดย่อ: การพัฒนาระบบการเพาะเลี้ยงเนื้อเยื่อเพื่อการขยายพันธุ์และการอนุรักษ์พันธุกรรมอ้อย (*Saccharum officinarum* L.) ได้ศึกษาการเกิด somatic embryo โดยนำชิ้นส่วนของช่อดอกอ่อนอ้อยมาเลี้ยงบนอาหารเลี้ยงเนื้อเยื่อพืช อาหารชักนำให้เกิดแคลลัสที่ให้ผลการตอบสนองสูงสุด ประกอบด้วยอาหาร Murashige and Skoog (MS) ที่มี 2,4-dichlorophenoxyacetic acid (2,4-D) 10-15 µM น้ำมะพร้าวอ่อน 50 มล./ล. casein hydrolysate (CH) 500 มก./ล. และน้ำตาล 6 % (w/v) การเพิ่มปริมาณแคลลัสทำได้โดยนำแคลลัสที่ได้จากอาหารชักนำย้ายไปเลี้ยงบนอาหาร MS ที่มีความเข้มข้นของ 2,4-D ลดลงเหลือ 5 µM มีผลให้แคลลัสสามารถเพิ่มปริมาณสูงสุด แคลลัสสามารถชักนำให้พัฒนาเป็นต้นอ่อนบนอาหาร MS ที่ประกอบด้วยปุ๋ยกล้วยไม้สูตร 21-21-21 อัตรา 0.5 ก./ล.และน้ำตาล 6 % (w/v) โดยไม่มีสารกระตุ้นการเจริญเติบโตพืช ต้นอ่อนสามารถเกิดรากได้เองบนอาหารสูตรนี้

คำสำคัญ: callus proliferation, induction percentage, ช่อดอกอ่อน, การขยายพันธุ์พืช

ABSTRACT: *In vitro* regeneration system for micro-propagation and cryopreservation of sugarcane (*Saccharum officinarum* L.) has been established. Somatic embryogenesis of sugarcane has been done using immature inflorescence segments as explants. The highest induction percentage for embryogenic callus was obtained from Murashige and Skoog (MS) basal medium supplemented with 10-15 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 50 ml/l coconut water (CW), 500 mg/l casein hydrolysate (CH), and 6 % (w/v) sucrose. For embryogenic callus proliferation, the lower concentration of 2,4-D (5 μM) displayed the highest callus growth rate (fresh weight basis). Embryogenic callus developed to plantlets on MS basal media containing 0.5 gm/l orchid fertilizer (21-21-21) and 6 % (w/v) sucrose without plant growth regulator. Plantlets could generate roots on this development media without plant growth regulator.

Keywords: callus proliferation, immature inflorescence, induction percentage, plant regeneration

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Introduction

Sugarcane (*Saccharum officinarum* L.) has been cultivated in about 60 countries in the tropics for production of sugar and bio-ethanol. Thailand ranks the second place for export of raw sugar to the world market. Lack of high quality planting materials and plant disease problem are the major constraints of sugarcane production in the country. To overcome these constraints, planting materials should be disease-free, and plant propagation system should be rapid to generate sufficient seed stock to the farmers. Micro-propagation system through plant tissue culture provides these solutions for rapid and sufficient planting materials without disease contamination. Somatic embryogenesis is a pathway to induce regeneration of plantlets from *in vitro* culture (Jimenez, 2001). The method has several applications including germplasm conservation through cryopreservation (Chanprame et al., 1993), regeneration of plantlets for synthetic seeds and production of disease-free seed stock for commercial production of crops (Litz and Gray, 1995). The technology of somatic embryogenesis is now mature enough for several crops such as carrot, soybean, cotton, and papaya (Litz and Gray, 1995). Unfortunately, the technology for somatic embryogenesis for sugarcane needs further investigation due to genotype specific (Gill et al., 2004; Taylor, 1992). The objective of this study was to establish the protocol for somatic embryogenesis of sugarcane using immature inflorescence segments as explants. The results are valuable of sugar industry for both sugarcane genetic management and planting material production.

Materials and Methods

Induction phase

Two sugarcane varieties, Khon Kaen 3 and U-Thong 8, used in these experiments were collected from 12-month-old plants (kindly provided by Khon Kaen Field Crop Research Center and Suphan-Buri Agricultural Research and Development Center). Fresh immature inflorescence segments were surface sterilized by Tween 20 and were washed several times by sterilized water. Finally, the plant materials were then surface sterilized by 20 % Clorox® (sodium hypochlorite, as available chlorine 5.25 % w/w) and were washed by sterilized water. The outer leaves were removed gently to avoid tissue damage. The immature inflorescence segments (approximately 4-6 inches long) were immerse into sterilized water supplemented with 100 mg/l Nystatin (*Phyto*Technology Laboratories™, KS, USA) and 50 mg/l Cefotaxime (*Phyto*Technology Laboratories™) to prevent contamination of microorganism. The innermost immature inflorescence were then cut into 5-10 mm long and cultured on induction media (IM). The induction media composed of Murashige and Skoog (MS) basal medium supplemented with various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D). In addition, various types of substances (coconut water (CW) and casein hydrolysate (CH)) were added to the IM media to investigate the promotion effect of these substances. Six percent (w/v) of sucrose was added to the media. The media were solidified by 0.3 % (w/v) Phytagel®. The media were adjusted to pH 5.7 and were autoclaved at 20 lb/sq.in. for 20 minutes. Filter sterilization of 200 mg/l ascorbic acid was

necessary for browning prevention. The explants were cultured in the dark environment in culture room at 25 ^{O}C for 4 weeks without changing the media. Theinduction percentages were recorded after 4 weeks of culture. The experimental design was the Completely Randomized Design (CRD) with 4 replications. For each treatment, 80 explants were used. The datawere statistically analyzed using IRRISTAT program. Raw data in percentage were transformed to arcsin squareroot of percentage before analysis. The experiments were repeated at least twice.

Proliferation phase

After 4 weeks in induction media (results from 1), the embryogenic callus were move to the proliferation media (PM). The PM composed of MS basal media containing various concentrations of 2,4-D. The others compositions were the same as described in (1), including 6 % (w/v) sucrose, 50 ml/l CW and 500 mg/l CH. The cultures were maintained in dark condition. Embryogenic callus from 10-15 µM 2,4-D, 50 ml/l CW and 500 mg/l CH were used in this experiment. For each treatment, 40 clumps were employed. Each clump was approximately 0.5 cm diameter. Proliferation performance was determine by fresh weight (FW) increment of callus clump after 3 or 6 weeks of culture (times compared to the initial FW).

$$\text{Growth increment (times)} = \frac{\text{FW at 3 or 6 weeks after culture-FW at initial experiment}}{\text{FW at initial experiment}}$$

Development phase

The cultures (from proliferation phase) were transferred to development media (DM) for plantlet development. The DM comprised of MS basal media supplemented with different concentrations of sucrose and 0.5 gm/l orchid fertilizer (21-21-21) without plant growth regulator. The media were solidified by 0.3 % (w/v) Phytagel®. The explants were cultured in culture room condition with cool white fluorescence light at 25 ^{O}C under a 16 hours light / 8 hours dark photoperiod. For each treatment, 80 clumps were employed. Each clump was approximately 0.5 cm diameter. The numbers of regenerated plantlets were record after 8 weeks of culture. The experimental design was the Completely Randomized Design (CRD) with 4 replications. The data were statistically analyzed using IRRISTAT program.

The experiments were carried out at the Office of Biotechnology Research and Development, Department of Agriculture, Thanyaburi, Pathum-Thani, between October 2010 to June 2012.

Results

After two weeks of dark incubation, the explants produced callus at the cut edge. The callus proliferated into opaque to yellowish clump (**Figure 1a**). The highest callus induction percentage (92-89%) were obtained from MS media containing 10-15 µM 2,4-D, 50 ml/l CW and 500 mg/l CH (**Table 1**). Adding CW enhance callus induction percentage, treatment 1 (control) vs treatment 2-5, (**Table 1**). Without CW and CH, callus induction percentage of these two varieties were lower than those supplemented with CW and CH. In combination with CW, addition of CH into the IM displayed the additive effect to enhance induction percentage.

Table 1 Induction of embryogenic callus (percentage) on different media compositions.

Media composition	Embryogenic callus (%)	
	Khon Kaen 3	U-Thong 8
1. MS + 10 µM 2,4-D (control)	29 d	24 e
2. MS + 10 µM 2,4-D + 50 ml/l CW	54 bc	58 bc
3. MS + 15 µM 2,4-D + 50 ml/l CW	60 b	52 c
4. MS + 20 µM 2,4-D + 50 ml/l CW	41 c	39 d
5. MS + 25 µM 2,4-D + 50 ml/l CW	45 c	49 cd
6. MS + 10 µM 2,4-D + 50 ml/l CW + 500 mg/l CH	92 a	87 a
7. MS + 15 µM 2,4-D + 50 ml/l CW + 500 mg/l CH	89 a	83 a
8. MS + 20 µM 2,4-D + 50 ml/l CW + 500 mg/l CH	70 b	69 b
9. MS + 25 µM 2,4-D + 50 ml/l CW + 500 mg/l CH	64 b	70 b
CV (%)	36	29

Means in the same column followed by the same letter(s) are not significantly different at 0.05 probability level by Duncan's New Multiple Range Test (DMRT).

MS stands for Murashige and Skoog, CW stands for coconut water and CH stands for casein hydrolysate

The highest proliferation of embryogenic callus (2.9 times compare to the initial (start) weight) was obtained on MS media containing 5 µM 2,4-D and 6 % sucrose (**Table 2 and Figure 1b**). Higher concentration of 2,4-D (above 5 µM) resulted in the reduction of callus growth rate. At 5 µM 2,4-D, high concentration of sucrose (6 %) promoted callus proliferation (2.9 at 6 % sucrose vs 2.1 at 2 % sucrose and 2.2 at 4 % sucrose). However, the difference between the media containing 5 µM and 10 µM of 2,4-D for callus proliferation was not significantly difference. In addition, the sugarcane varieties performed differently for callus proliferation, Khon Kaen 3 performed better than U-Thong 8.

For plantlet induction, the highest numbers of plantlets per callus clump were obtained from MS basal media containing 0.5 gm/l orchid fertilizer (21-21-21) and 6 % sucrose (**Table 3**). After being moved to light condition, callus turned to yellowish green and the surface area of each clump expanded (**Figure 1c**). High numbers of plantlets were obtained on the expanded surface area. Moreover, plantlets could generate root in the same media without plant growth regulator (**Figure 1d**).

Table 2 Callus proliferation (times compared to initial weight) of embryogenic callus on different media.

Media composition	Growth increment of embryogenic callus			
	3 weeks		6 weeks	
	Khon Kaen 3	U-Thong 8	Khon Kaen 3	U-Thong 8
1. MS + 10 µM 2,4-D + 2% sucrose (control)	1.5 bc	1.0 b	3.0 b	2.1 b
2. MS + 5 µM 2,4-D + 2% sucrose	2.1 b	1.0 b	3.2 b	2.3 b
3. MS + 15 µM 2,4-D + 2% sucrose	1.1 c	0.9 b	2.1 c	1.3 c
4. MS + 20 µM 2,4-D + 2% sucrose	1.2c	0.9 b	2.1 c	1.3 c
5. MS + 5 µM 2,4-D + 4% sucrose	2.2 b	1.4 a	3.1 b	2.2 b
6. MS + 15 µM 2,4-D + 4% sucrose	1.3 c	0.8 b	2.0 c	1.3 c
7. MS + 20 µM 2,4-D + 4% sucrose	1.3 c	0.8 b	2.0 c	1.2 c
8. MS + 5 µM 2,4-D + 6% sucrose	2.9 a	1.6 a	5.8 a	3.1 a
9. MS + 15 µM 2,4-D + 6% sucrose	1.2 c	1.0 b	2.2 c	1.4 c
10. MS + 20 µM 2,4-D + 6% sucrose	1.2 c	1.0 b	2.1 c	1.5 c
CV (%)	36	41	38	32

Means in the same column followed by the same letter(s) are not significantly different at 0.05 probability level by Duncan's New Multiple Range Test (DMRT).

Table 3 Numbers of shoots per callus clump on different media composition.

Media composition	No. of shoots per callus clump			
	Khon Kaen 3	Responding percentage[1]	U-thong 8	Responding percentage
1. MS + 2% sucrose (control)	4 c	85	6 c	84
2. MS + 2% sucrose + orchid fertilizer	8 bc	89	9 bc	89
3. MS + 4% sucrose	5 c	89	6 c	86
4. MS + 4% sucrose + orchid fertilizer	10 b	92	14 b	92
5. MS + 6% sucrose	8 bc	90	9 bc	89
6. MS + 6% sucrose + orchid fertilizer	18 a	92	22 a	87
CV (%)	29		26	

Means in the same column followed by the same letter(s) are not significantly different at 0.05 probability level by Duncan's New Multiple Range Test (DMRT).

[1]responding percentages were the percentages of clump that generate shoots.

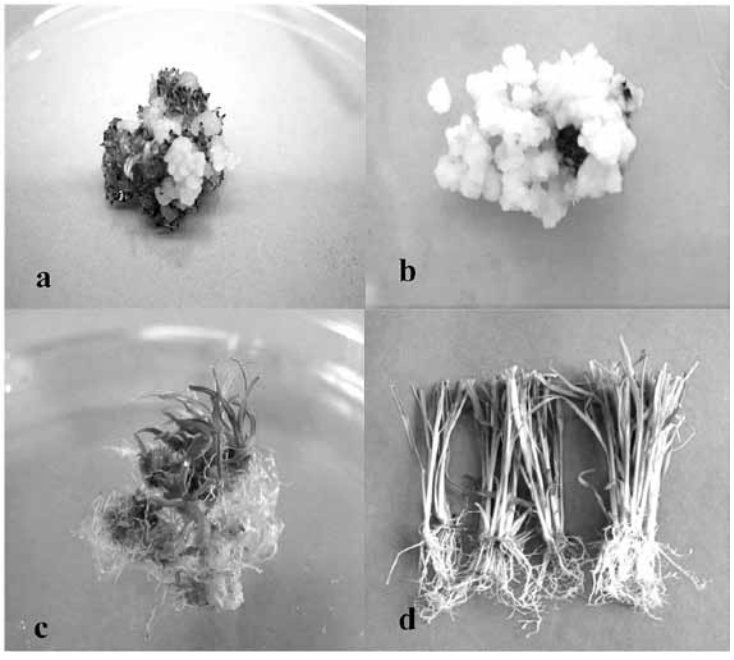

Figure 1a. Induction of embryogenic callus on MS media + 10 µM 2,4-D, 50 ml/l CW and 500 mg/l CH.
1b. Proliferation of embryogenic callus on MS media + 5 µM 2,4-D, 50 ml/l CW and 500 mg/l CH.
1c. Plantlets development on MS media + 0.5 gm/l orchid fertilizer (21-21-21) and 6 % (w/v) sucrose.
1d. Rooting of plantlets on MS media + 0.5 gm/l orchid fertilizer (21-21-21) and 6 % (w/v) sucrose.

Discussion

Reliable and reproducible protocols of micro-propagation techniques are required for routine work in genetic conservation and effective production of sugarcane planting material. Therefore, the objective of this research project was to establish the most suitable protocol for callus proliferation and plant regeneration of sugarcane using immature inflorescence as a start explant in tissue culture. The use of immature inflorescence in this investigation was because there were many examples of success in somatic embryogenesis studies in sugarcane (Liu, 1993; Martinez-Montero et al., 1998; Desai et al., 2004).

Selection of plant growth regulator was also based on previous searching, 2,4-D was necessary for induction of embryogenic callus in sugarcane (Martinez-Montero et al., 1998; Snyman et al., 2000; Gill et al., 2004; Ali et al., 2007) and, therefore, 2,4-D was varied for 4 levels (10-25 µM) in this study to investigate the optimum concentration. Coconut water and casein hydrolysate were also included in the media as these substances are commonly used in plant tissue culture and they have been effective in many plant species.

At induction phase, the media containing 10-15 µM 2,4-D with CW and CH had the highest induction percentage. At this 2,4-D concentrations, explants displayed less browning effect. The explants turned brown after two weeks of culture at the higher concentrations of 2,4-D (above 15 µM) that resulted in cell dead. Therefore, optimum concentration of 2,4-D is required for optimum

growth. In addition, explants cultured in the dark condition displayed less browning effect compare to in the light condition (data not shown). This results showed that CW and CH enhanced callus induction percentage. It is concluded that, at induction phase, the media should not contain 2,4-D higher than 15 µM. CW and CH should be included in the media and the culture should be maintained under dark condition.

The results supported previous finding for the effects of CW and CH in promoting growth in sugarcane (Ho and Vasil, 1982) and for the effect of 2,4-D at low concentrations on proliferation of embryogenic callus (Ho and Vasil, 1983; Gill et al., 2004). However, sugarcane genotypes respond differently to these factors (Taylor, 1992; Gill et al., 2004). This study also showed that callus proliferation performance was different between Khon Kaen 3 and U-Thong 8.

In previous investigation, plantlets of sugarcane could develop on MS media without plant growth regulators (Martinez-Montero et al., 1998). In this investigation, however, addition of 0.5 gm/l orchid fertilizer (21-21-21) enhanced growth of plantlets. Plantlets that received orchid fertilizer were dark green at leaves and stem. The results were then conclusive for the benefit of orchid fertilizer in promoting growth of sugarcane in tissue culture. Some genotypes might require plant growth regulators but growth regulators might be not necessary for some genotypes. High level of sucrose (6 %) also promoted plantlet development, and the results were in good agreement with previous report using 6 % of sucrose (Ho and Vasil, 1982).

In this study, the plantlets could generate root in the development media, transferring of plantlets to rooting media was not required. Root development in development media would be due to high concentration of sucrose and the lack of cytokinin. The present of cytokinins in the development media hindered root formation in sugarcane (Gill et al., 2004), and high level of sucrose promoted root formation (Gill et al., 2004).

In this investigation, we report a reliable and reproducible protocol for regeneration of embryogenic callus which can be used in cryopreservation research and production of seed stock for farmers. Similar investigation using different protocol was also reported (Chanpramme et al., 1993; Martinez-Montero et al., 1998). The results support previous findings and also contribute to the advancement in sugarcane research.

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