THE TISSUE CULTURE OPTIMIZATION FOR *AMORPHOPHALLUS ONCOPHYLLUS* CELL SUSPENSION FOR KONJAC GLUCOMANNAN PRODUCTION

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ABSTRACT

The *in vitro* shoots from leaf bulbils of elephant yam (*Amorphophallus oncophyllus*) were cultured on MS basal medium supplemented with 0, 0.5, 1.0 and 2.0 mg/l 6-benzylaminopurine (BAP). The highest average multiple shoots of 6.45 shoots/segment were obtained from MS basal medium supplemented with 2.0 mg/l BAP. For callus induction, petiole and leaf segments of elephant yam were cultured on MS basal medium with varying concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D), from 0, 0.25, 0.5, 1.0 and 2.0 mg/l under dark and light conditions at controlled temperature of about 25±2 °C for 60 days. As a result, 55% and 72.5% of callus formation were obtained from MS basal medium supplemented with 0.5 mg/l 2,4-D under dark and light conditions, respectively. Additionally, MS basal medium with 0.25 mg/l α-naphthaleneacetic acid (NAA) induced the friable calli from petiole and leaf segments with the callus formation of 80% under light condition. However, the highest percentage (60%) of friable calli induction was obtained from the differentiated compact calli under light condition and cultured on MS basal medium supplemented with 0.75 mg/l 2,4-D. Moreover, the friable calli were multiplied under dark condition in MS basal medium with 0.25 mg/l NAA and found to successfully form small-size cell suspension after several 7-day interval subculture into MS liquid medium with 0.25 mg/l NAA and 20 g/l sucrose. Finally, this small-size cell suspension will be optimized further in a bioreactor, for the production of konjac glucomannan.

Keywords: konjac glucomannan; elephant yam; *Amorphophallus oncophyllus*; cell suspension culture

INTRODUCTION

The elephant yam plant (*Amorphophallus*), a perennial herbaceous herb, is a member of the Araceae family. It grows on mountain or hilly areas in subtropical regions mainly in the South East of Asia. There are many species of elephant yam plant in the Southeast Asia that belong to the *Amorphophallus*, e.g. *A. konjac* C. Koch, *A. rivierii, A. bulbifer* and *A. oncophyllus*. The plant has high content of glucomannan in subterranean corms and it has been used as food and food additives in China and Japan for more than 1000 years. Konjac glucomannan (KGM) is a neutral polysaccharide derived from the konjac tubers. It is

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composed of β-1,4 linked D-mannose and D-glucose with the mannose/glucose ratio of 1.6:1 [1]. In Thailand, we found the elephant yam plant up to 68 species. As characterizing glucomannan biochemically, *Amorphophallus oncophyllus* Prain., was reported to be highly potential for use in the industry [2]. Glucomannan is a hydrocolloidal polysaccharide that serves as a soluble dietary fiber and contains no calories. When taken orally glucomannan can absorb water up to 100 times of its weight and induce a feeling of fullness. Konjac glucomannan has several other health benefits serving as a weight loss aid. Recent studies showed that glucomannan may decrease total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C), without changing the high density lipoprotein cholesterol (HDL-C) level. At the same time, no adverse effect on the absorption of iron, calcium, copper and zinc was found in adults with insulin-resistance syndrome or type II diabetes or in obese children [3, 4]. In terms of the benefit to the gastrointestinal tract, glucomannan consumption has been shown to enhance the growth of bifidobacteria, which are the beneficial bacteria in the gut and to relieve constipation without causing diarrhea or bloating. In addition, clinical studies showed that glucomannan helps control or lowers blood sugar level after meal probably by slowing the absorption of glucose from the gut. The Food Chemicals Codex in the United States only listed konjac flour as food additives [5]. Moreover, characters of low cost, excellent film-forming ability, good biocompatibility, biodegradability and gel-forming properties entitle KGM to be a novel polymer material. This makes natural KGM promising for many applications in various fields like packaging and preservative materials [6], and control releasing materials [7].

However, the production of KGM industrially is quite limited due to the shortage of raw material supplied naturally and agriculturally [2]. Although the tissue culture technique has possibly been applicable, a growing time necessary for plant cultivation is economically obvious [8]. The aim of this study is focused on the production of KGM from *Amorphophallus oncophyllus* by using cell suspension culture through fermentation technology that is alternatively applicable in an industrial scale.

MATERIALS AND METHODS

Materials and culture media

Explants of genus *Amorphophallus oncophyllus* such as leaf bulbils, tuber, bud and sucker were rinsed under running tap water and then surface sterilized by successive immersion for 5 min in 70% (v/v) ethanol, and 15, 20 and 25 min in 10, 15, 20 and 25 % (v/v) HAITER® (sodium hypochlorite (NaClO) as available chlorine 6% w/w) solution containing two drops of Tween-20. After sterilization and rinsing with sterile water three times, the explants were sliced into segments with a scalpel and incubated on MS agar medium.

MS culture medium [9] was used thoroughly in this study with slight modifications by supplementing 3% (w/v) sucrose and solidified using 0.8% (w/v) agar. The pH was adjusted to 5.8 before autoclaving at 121°C for 15 min.

The *in vitro* shoot culture conditions

The induced shoots and multiple shoots prepared from leaf bulbils and sucker of elephant yam were cultured on MS agar medium (pH 5.8) supplemented with varying concentration of 6-benzylaminopurine (BAP) 0, 0.5, 1.0 and 2.0 mg/l. The cultivations were conducted under light condition 16 h/day at controlled temperature of 25±2 °C for 60 days.

Induction of callus

To induce callus from petiole and leaf segments of multiplied shoots, the explants were cultured on MS agar medium (pH 5.8) with and without supplementation of varying

concentrations of 0.25, 0.50, 1.00 and 2.00 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) or α-naphthaleneacetic acid (NAA) under the light-dark condition at controlled temperature of 25±2 °C for 60 days. The callus induced from petiole and leaf segments were routinely subcultured at monthly intervals.

Induction of friable calli

To induce friable calli, the compact calli were subcultured to fresh MS basal medium (pH 5.8) with varying concentrations of 0.25, 0.50, 1.00 and 2.00 mg/l 2,4-D for 60 days.

Cell suspension culture

The friable calli were transferred into MS liquid medium (pH 5.8) containing 20 g/l sucrose supplemented with varying concentrations of 0.1, 0.75 mg/l 2,4-D or 0.25, 2.0 mg/l NAA. They were subcultured every 7 days for 60 days and cultured under the dark condition at 25±2 °C and 120 rpm shaking.

RESULTS AND DISCUSSION

Preparation of the sterilized tissue of *Amorphophallus oncophyllus*

The surface sterilization of elephant yam tissue from leaf bulbils, tuber, bud and sucker was carried out by using various sodium hypochlorite concentrations and times under culture on MS basal medium, 16 h/day light conditions and 25±2 °C for 7 days. The survivals of 82.5 and 82.5% were obtained from sucker and leaf bulbils with the use of 20/20 and 20/25 %HAITER®/min, respectively, (Table 1). Therefore, the sucker and leaf bulbils of the elephant yam were used for the preparation of sterilized tissues to induce the shoots under the optimal conditions mentioned above.

Effect of benzylaminopurine (BAP) on the induction of shoots

The sterilized elephant yam tissues prepared from leaf bulbils and sucker were cultured on MS basal medium with varying concentrations of 0, 0.5, 1.0 and 2.0 mg/l BAP. It was found that the small shoots were induced from the sterilized sucker within 45 days (Fig. 1A). After being transferred onto fresh MS agar medium with 2.0 mg/l BAP, the highest average multiple shoots of 6.45 shoots/segment were formed (Fig. 1B), after incubation under 16 h/day light condition at controlled temperature of 25±2 °C for 60 days (Table 2).

Effect of two phytohormones on the induction of callus

The phytohormones 2,4-dichlorophenoxy acetic acid (2,4-D) and α-naphthaleneacetic acid (NAA) were investigated for the induction of callus. The petiole and leaf segments of elephant yam were cultured on MS medium with and without 2,4-D or NAA of varying concentrations. It was observed that 2,4-D played an important role in inducing the callus formation of *Amorphophallus oncophyllus* (Fig. 1C). As a result, the 2,4-D concentration of 0.5 mg/l was found to be optimal for the callus formation of 55% and 72.5% from leaves under dark and light conditions, respectively (Table 3). These calli were able to develop into the complete callus (Fig. 1D). When cultured on MS medium with NAA, the formation of callus was also induced (Table 4), especially for the roots which were attained at higher NAA concentration. Moreover, MS basal medium with 0.25 mg/l NAA was found to induce the friable calli from petiole and leaf segments optimally with 80% callus formation under light condition.

Table 1: The survival of various *Amorphophallus oncophyllus* tissues treated successively with 70% (v/v) ethanol (for 5 min) with varying concentrations of sodium hypochlorite and time.

HAITER® (% v/v)	Time (min)	% Survival			
		Leaf Bulbils	Tuber	Bud	Sucker
10	15	0.0	0.0	0.0	2.5
10	20	0.0	0.0	0.0	2.5
10	25	0.0	0.0	2.5	5.0
15	15	7.5	0.0	12.5	7.5
15	20	12.5	2.5	17.5	10.0
15	25	20.0	5.0	20.0	20.0
20	15	27.5	10.0	20.0	37.5
20	20	50.0	17.5	35.0	82.5
20	25	82.5	35.0	42.5	80.0
25	15	50.0	20.0	35.0	70.0
25	20	57.5	30.0	30.0	57.5
25	25	22.5	32.5	27.5	45.0

Note: Explants were rinsed with tap water, immersed in 70% (v/v) ethanol and HAITER® (sodium hypochlorite (NaClO) as available chlorine 6% w/w) solution with Tween-20, washed with sterile water thrice, sliced into segments and incubated on MS agar medium with an initial pH 5.8 under 16-h/day light condition at 25±2 °C for 7 days.

Table 2: Effect of 6-benzylaminopurine (BAP) on the induction of elephant yam shoots *in vitro*.

BAP (mg/l)	Average Shoot Number
0.0	1.65
0.5	2.00
1.0	3.80
2.0	6.45

Note: The elephant yam shoots prepared from leaf bulbils and sucker were cultured on MS agar medium supplemented with BAP under 16 h/day light condition at 25±2 °C for 60 days.

Table 3: Effect of 2,4-D on the formation of compact callus from petiole and leaf segments under light and dark conditions.

2,4-D (mg/l)	Compact Callus Formation (%)					
	Dark Condition			Light Condition		
	Leaves	Middle Part	Basal Part	Leaves	Middle Part	Basal Part
0.00	30.0	10.0	15.0	22.5	12.5	17.5
0.25	42.0	20.0	30.0	47.5	30.0	30.0
0.50	55.0	40.0	40.0	72.5	55.0	45.0
1.00	50.0	35.0	37.5	55.0	35.0	37.5
2.00	30.0	20.0	12.5	37.5	15.0	17.5

Note: The explants were cultured on MS agar medium supplemented with and without 2,4-dichlorophenoxy acetic acid (2,4-D) under the 16 h/day light or dark condition at 25±2 °C for 60 days.

Table 4: Effect of NAA on the formation of compact callus from petiole and leaf segments under light condition.

NAA (mg/l)	Compact Callus Formation (%)		
	Leaves	Middle Part	Basal Part
0.25	70	80	70
0.50	80	90	80
0.75	80	90	80
1.00	80	90	80
2.00	80	90	80

Note: The explants were cultured on MS agar medium supplemented with and without α-naphthaleneacetic acid (NAA) under the 16 h/day light condition at 25±2 °C for 60 days.

Effect of 2,4-D on the induction of friable calli

For using 2,4-D to induce the friable calli, the callus of elephant yam was cultured on MS medium supplemented with 0.25, 0.5, 1.0 and 2.0 mg/l 2,4-D. The 2,4-D concentration of 0.5 mg/l was optimal for 10% formation of friable calli under cultivation for 60 days. It was subcultured into fresh MS basal medium with 0.5 and 0.75 mg/l 2, 4-D. The number of friable calli increased to 60% in MS basal medium with 0.75 mg/l 2, 4-D, which was cultured for 60 days under light condition (Fig. 1E).

Establishment of cell suspension culture

The friable calli mentioned above were transferred into liquid MS basal medium (pH 5.8) containing 20 g/l sucrose with varying concentrations of 0.10, 0.75 mg/l 2,4-D and 0.25, 2.00 mg/l NAA. They were subcultured every 7 days for 60 days and continuously maintained at 25±2 °C on the shaker at 120 rpm in the dark. It showed that the friable calli were able to grow and multiply (Fig. 1F). So far, the small-sized cells were developed after several transfers in MS basal medium with 0.25 mg/l NAA (Fig. 2).

CONCLUSIONS

The elephant yam was successfully induced for compact and friable calli in this work. MS basal medium with 0.5 and 0.75 mg/l 2,4-D was found to be able to differentiate the compact calli into friable calli. However, the highest percentage (60%) of friable calli induction was obtained from the differentiated compact calli under light condition when cultured on MS basal medium supplemented with 0.75 mg/l 2, 4-D. Moreover, the friable calli were multiplied under dark condition on MS basal medium with 0.25 mg/l NAA and found to successfully form small-sized cell suspension after several 7-day subculture on liquid MS medium with 0.25 mg/l NAA and 20 g/l sucrose. Finally, this small-sized cell suspension will be optimized further in a bioreactor, for the production of KGM.

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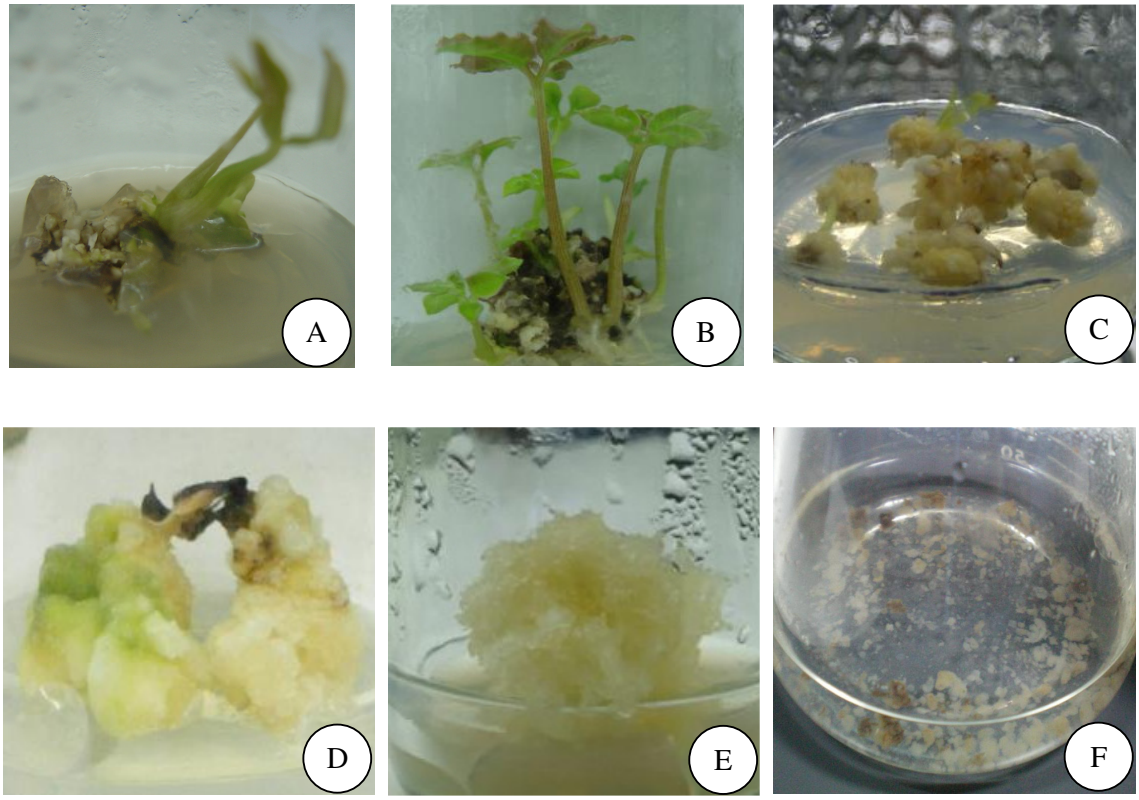

Figure 1: Plant regeneration via adventitious sucker of elephant yam. (A) The small sucker of elephant yam cultured on MS medium with 2.0 mg/l BA for 45 days. (B) The average shoot numbers of elephant yam cultured on MS medium with 2.0 mg/l BA for 60 days. (C) The formation of callus cultured on MS medium with 0.5 mg/l 2,4-D. (D) The callus developed into a complete callus. (E) The friable calli of elephant yam. (F) The friable calli cultured in MS basal medium containing 20 g/l sucrose and 0.25 mg/l NAA.

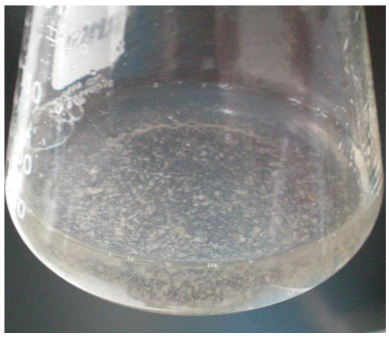

Figure 2: The cell suspension of *Amorphophallus oncophyllus* in MS basal medium supplemented with 0.25 mg/l NAA.

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