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RESEARCH ARTICLES

CONDITIONS FOR ISOLATION AND CULTURE OF *ARANDA* CHARK KUAN MESOPHYLL PROTOPLASTS

KAMNOON KANCHANAPOOM AND PATWADEE TONGSEEDAM

Department of Biology, Faculty of Science, Prince of Songkla University, Haad-Yai, Songkla 90112, Thailand.

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ABSTRACT

Aranda *Chark Kuan buds were cultured on Vacin and Went (1949) agar medium for 1 month. After removing the leaves and the apex from the bulging bud and transferring it to a liquid medium, protocorm-likebodies (plb) were obtained. When these plb were transferred onto the same agar medium, complete plantlets developed. Protoplasts were isolated from young leaves (1-3 cm) of these plantlets. One gram fresh weight of the leaves was digested with 10mlofenzyme solution containing 2% (w/v) Cellulase Onozuka R-10,0.7 Msorbitol,10mM* $CaCl_2.2H_2O$*, 10mM MES. The mixture of leaves and enzyme solution was incubated at 30°C for 7 hours on a gyrotary shaker with an agitation speed of 40 rpm. The average yield of isolated protoplasts was* $3.6x10^5$ *per gram fresh weight. The protoplast culture was investigated according to different types of media and culture methods. The best result found was* $1x10^5$ *initial protoplasts/ml cultured in Nagata and Takebe (1970) liquid medium supplemented with 1 mg/l 2,4-D (2,4-dichlorophenoxy acetic acid) and 1 mg/l BA* (N^6*-benzyladenine) under dark condition. After 3 days in culture, wall formation was clearly visible; however, cell division was not evident.*

INTRODUCTION

Orchids are outstanding horticultural and floral crops with large diversity in the family. They are conventionally propagated by either splitting the pseudo bulbs or top cutting to initiate the axillary buds to form new shoots. To date plant regeneration by tissue culture of orchids is common[1-5]. Mass clonal propagation of orchid is adequate and satisfactory. However, a lot of research to improve orchid varieties needs to be conducted. Protoplast culture and somatic hybridization is another valuable tool with potential impact not only for academic interest but also of commercial importance. Unfortunately, reports on orchid protoplast are very few [6-11]. In this paper, we describe our attempt to isolate and culture *Aranda* Chark Kuan protoplast.

MATERIALS AND METHODS

Plant material

Aranda Chark Kuan used for isolation of protoplasts were grown aseptically following the methods described by Goh [12]. Young plantlets were subcultured once a month and used as sources of protoplasts.

Protoplast isolation

Protocorms, roots and leaves were used for protoplast isolation. Protocorms and roots of aseptic *Aranda* Chark Kuan were chopped into pieces but leaves of size 1-3 cm were excised at the bases and cut longitudinally into two pieces. One gram fresh weight of all plant materials were used per 10 ml of enzyme solution. Three kinds of enzymes were tested: Cellulase OnozukaR-10 (Yakult Honsha Co., Ltd. Lot # 201050), Driselase (Kyowa Hakko Co., Ltd. Lot#4111and Pectinase (ICN BiochemicalLot#18832). All enzymes were dissolved in 0.7 M sorbitol,10mM $CaCl_2.2H_2O$ and 10 mM MES. The leaf-enzyme mixture was placed on a rotary shaker (40 rpm; 30°C). After 7h incubation, protoplasts were seived through a 43 µm mesh stainless steel screen to remove any clumps of undigested cells and debris. The filtered protoplasts were centrifuged at approximately 40xg for 5 min. The pellet was resuspended and washed twice in 0.7 M sorbitol and finally resuspended in 0.6M sucrose solution. The floating protoplasts were collected and counted using an AO Bright-Line hemacytometer slide at 100x.

Protoplast culture

Protoplasts were cultured either under 16 h light regime or in complete darkness at a density of $1x10^5$ /ml in 55 x15 mm petri dishes. The protoplasts were cultured using various methods: thin layer of liquid culture, thin solid culture, semi-solid culture and sitting drop culture.

Assessment of protoplast viability and wall formation

The viability of freshly isolated protoplasts was monitored using fluorescein diacetate (FDA). Solution of FDA (5 mg/ml acetone) was added to protoplast suspension at 0.1% (v/v). After 5-10 min incubation, protoplasts were examined using an Olympus BH2-RFL microscope with an epi-fluorescence attachment. The light source for fluorescence microscopy was an OSRAM HBO 100W high pressure mercury lamp. For FDA,the filter combinations were dichroic mirror, exciter filter unit blue (B) and barrier filter L-435. The presence of wall material was monitored using calcofluor white (Sigma). An aliquot of 0.7M sorbitol solution containing 0.1%(w/v) calcofluor white was mixed with an equal volumeof protoplast suspension. For calcofluor white, the filter combinations were dichroic mirror, exciter filter unit ultraviolet (U) and barrier filter 0-515. The fluorescence of labelled protoplasts was photographed using photographic system Olympus model PM-10AD with Kodacolor ASA 400 films.

Culture media

Five culture media were evaluated. VW was as described by Vacin and Went[13], KC (Knudson C)[14], MS (Murashige and Skoog)[15] , B5 (Gamborg)[16] and NT (Nagata and Takebe)[17]. All media were supplemented with0.44mg/l 2,4-D (2,4-dichlorophenoxy acetic acid) and 0.22 mg/l BA (N^6-benzyladenine), except for NT medium, which was supplemented with1 mg/l 2,4-D and 1mg/l BA. Sorbitol was used as an osmoticum unless otherwise stated. All media were filter-sterilized and stored at 25°C prior to use.

RESULTS

Aranda Chark Kuan buds swelled and became semi-spherical during the first month of culture on an agar medium. Removal of leaf primordium and apex was necessary before transferring to a liquid medium to induce plb. When these plb were subcultured onto the same solid agar medium , they differentiated into complete plantlets (Fig.1).

Light seems to be a critical factor that has deleterious effects on both isolation and culture of protoplasts. *Aranda* Chark Kuan donor plantlets which had been used for isolation were kept in the dark for 24h prior to use and during degradation of cell walls. This resulted in higher yield of protoplast than in the case of untreated plants. Results are not shown since they are in agreement with those already published[10,18].

Protoplasts were isolated from leaves in 2% (w/v) cellulase for 5 h in several concentrations of sorbitol. After 1h in enzyme solution, mesophyll cells plasmolysed and plasma membrane contracted away from the wall (Fig.2). Released protoplasts were first observed after 3 h incubation and continued for up to 11 h. The highest number of protoplasts obtained was $2x10^5$ per g.f. wt. in 0.7M sorbitol (Table1). Therefore, we routinely used 0.7M sorbitol as an osmoticum for isolation. To evaluate sources of protoplasts; protocorms, leaves, and roots of young *Aranda* Chark Kuan were tested in 2% (w/v) cellulase, 0.7M sorbitol. It was found that leaves provided the highest release of protoplasts ($3.4x10^5$ per g. f. wt.) compared to roots and protocorms (Table 2).

Mesophyll protoplasts showed various types of chloroplast distribution (Fig.3) and some protoplasts contained no chloroplasts. The size of mesophyll protoplasts ranged 40-90 μm in diameter and were more uniform in size (50-60 μm) from leaves 1-3 cm in length. Crystals could be seen in many preparations and apparently these crystals were from burst idioblasts. The freshly isolated protoplasts were of spherical shape and were yellow-green with FDA fluorescence and red with chlorophyll fluorescence indicating viability after release. Better yield of protoplasts was obtained when the leaf-enzyme mixture was incubated at 30°C than at 25°C (Table3). Many protoplasts ruptured at 60 rpm thus resulting in a lower yield. To determine the optimum type and time of enzyme action, various enzyme mixtures were used to test the release of protoplasts from mesophyll cells as shown in Table 4. It was found that for single enzyme, $3.6x10^5$ protoplasts per g.f. wt. was achieved from 2% cellulase at 7 h which was better than the highest yield of protoplasts obtained from enzyme combination of 1% cellulase and 1% pectinase ($2.8x10^5$ protoplast per g.f.wt. at 11 h). The sizes of leaf explants between 1-3 cm and longer than 3 cm showed no difference in number of isolated protoplasts. However, protoplasts released from leaves of size 1-3 cm

Table 1. *Aranda* Chark Kuan mesophyll protoplasts release from various sorbitol concentrations.

Sorbitol concentration(M)	**Protoplast yield ($x10^5$ per g. f. wt.) X±SD**	**Protoplast observation**
0.5	1.02±1.12	Normal,turgid
0.7	2.03±0.05	Normal,turgid
0.9	0.83±0.45	Crenate

Table 2. Protoplast isolation in 2% (w/v) cellulase, 0.7M sorbitol from various *Aranda* Chark Kuan sources.

Plant part	**Protoplast yield ($x10^5$ per g. f. wt.) X±SD**
Roots	1.66±1.15
Leaves	3.43±0.25
Protocorms	0.10±0.00

Table 3. Effect of incubation temperature on the release of protoplasts.

Temperature (°C)	**Protoplast yield ($x10^5$ per g.f.wt.) X±SD**
25	2.30±0.232
30	3.00±0.15

Table 4. Effect of type and concentration of enzymes on the isolation of *Aranda* Chark Kuan mesophyll protoplasts at different incubation times.

Type and conc. of enzyme	Protoplast yield ($x10^5$ per g. f. wt.) X±SD			
	5	7	9	11 (h)
1% Cellulase	1.50±0.36	1.00±0.00	0.90±0.40	1.10±0.38
2% Cellulase	1.70±0.24	3.60±0.25	1.80±0.50	2.60±0.23
3% Cellulase	0.10±0.49	0.19±0.10	0.70±0.34	0.23±0.10
1% Driselase	1.00±0.76	0.83±0.45	0.54±0.10	0.56±0.92
2% Driselase	0.89±0.15	0.88±0.20	0.80±0.10	0.30±0.47
3% Driselase	0.19±0.24	0.130±0.36	*	*
1% Cellulase and 1% Driselase	0.90±0.10	0.80±0.00	0.30±0.26	*
1% Cellulase and 1% Pectinase	1.00±0.56	0.70±0.787	1.80±0.50	2.50±0.50
1% Driselase and 1% Pectinase	1.80±0.15	2.00±0.20	1.90±0.85	2.80±0.22
1% Cellulase, 0.5% Driselase and 0.5% Pectinase	0.70±0.98	1.30±0.14	2.00±0.54	*

* Protoplast yield too low to be counted.

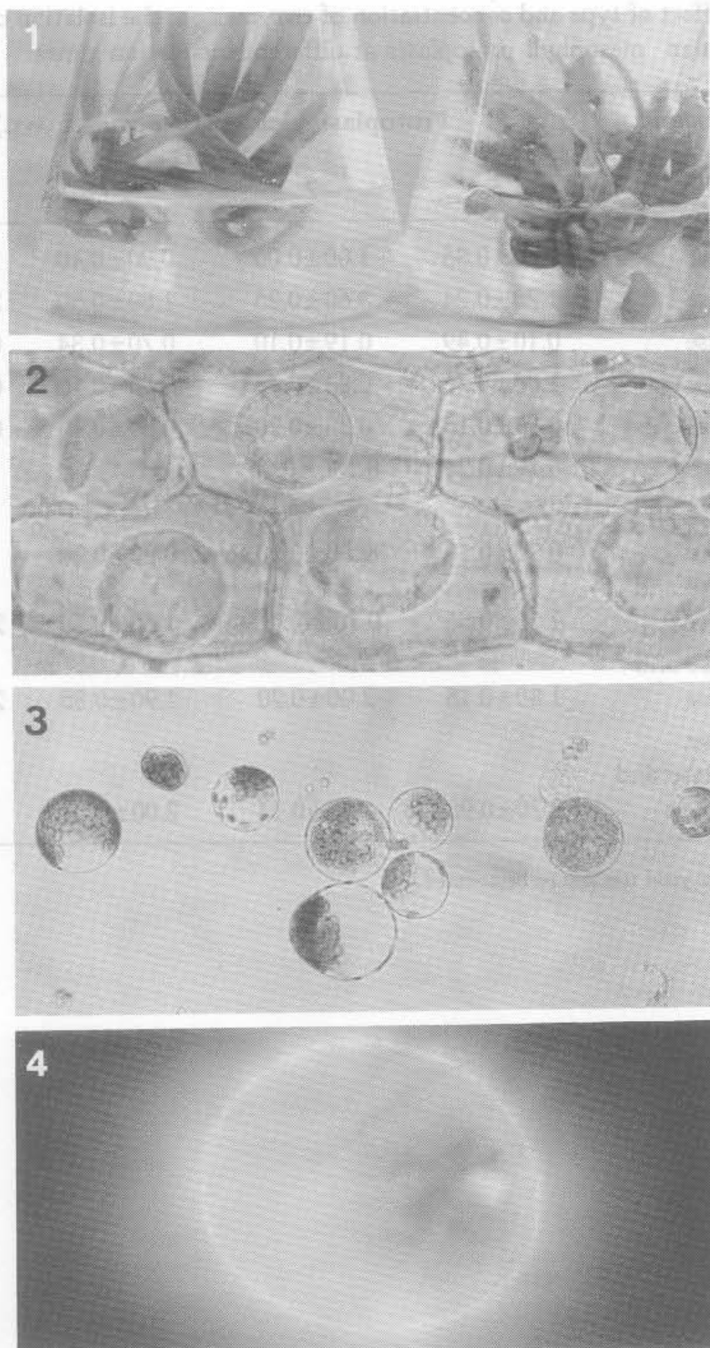

Fig. 1. Plant regeneration from plb on VW agar medium.

Fig. 2. Pre-plasmolysed mesophyll cells after 1h incubation in 2% cellulase, 0.7M sorbitol (x 165).

Fig. 3. Freshly isolated protoplasts from *Aranda* mesophyll cells (x 125).

Fig. 4. A protoplast-derived cell 3 d after culture stained with calcofluor white (ultraviolet illumination) (x 165

were small and had condensed chloroplasts with small vacuoles, while protoplasts from leaves longer than 3 cm gave protoplasts of greater size and with large vacuoles.

In the experiment using different culture methods for protoplast culture, a high survival of protoplast was found in the thin layer of liquid medium. Three days after culture in the light protoplasts in all media had lysed, chloroplasts turned brown and pelleted at the bottom of petri dishes. Under dark condition, protoplasts could survive in all media for several weeks but cell division was not evident. Assessments using FDA confirmed that 80-90% of the cultured protoplasts were viable. Other sugars such as sucrose, glucose, maltose and mannitol were substituted for 0.7M sorbitol. No significant differences in culture were evident among these sugars.

The best result for protoplast culture in our experiment was obtained from NT liquid medium. Assessments using calcofluor white made 3 days after culture in this medium indicated that wall formation was present but cell division had not occurred. (Fig.4). Regenerated cells persisted more than 4 weeks and subsequently became necrotic suggesting that NT liquid medium used in this study is not suitable for regeneration.

DISCUSSION

The balance of concentration of plant growth regulators in *Aranda* Chark Kuan bud changes due to the effect of wounding and the new balance promotes protocorm formation. Growth and proliferation of protocorms are usually carried out in liquid medium. For differentiation into complete plantlets they should be grown on solid medium. *Aranda* Chark Kuan budsin this study also showed the same pattern of development as other orchids[4,19].

The isolation conditions are extremely important for the release of protoplasts without impairing cell viability and to achieve maximum yield. Many factors, such as concentration of osmoticum, temperature, time of incubation, concentration and types of enzymes, have been shown to influence the quantity and quality of protoplast release. The concentration of sorbitol used was found to be effective. Preparation predominantly uninucleate of protoplasts was achieved possibly due to the 0.7 M sorbitol providing a prior-plasmolysis stage. It is often beneficial if the enzyme solution is slightly hypertonic since there is evidence that plasmolysis hastens wall degradation, perhaps by allowing enzyme attack from the inner surface of the wall[20]. Pais *et al*[21]. reported that 0.7 M mannitol gave the best results for the terrestrial orchid, *Barlia longibracteate*. This is in contrast to Koh *et al*[10]. who employed 0.4 M sucrose in the enzyme solution.

Among the hydrolytic enzymes used in this experiment, cellulase alone was found to be the most effective in releasing protoplasts from the leaves. However, as the enzyme concentration was increased, the yield of protoplast decreased. Cellulase concentration above 2% caused lysis of protoplasts, resulting in a drastic reduction in the yield of protoplasts. The same trend was seen on Driselase. Cellulase in combination with Driselase did not result in an increase of yield, while Koh *et al*[10]. reported that inclusion of 0.5% Driselase into the enzyme mixture of 1.5% Cellulase Onozuka R-10and 0.5% Macerozyme increased the yield of *Aranda* hybrids mesophyll protoplasts. The probable reason for this is that the enzyme was not well purified and this affected yield and viability since the crude

enzyme contains various impurities which have a harmful effect on the protoplasts.

Several types of chloroplast distribution and idioblasts containing stacked raphides are common in *Aranda* and different orchid taxa [9,11,12,13]. Lysis is a major problem associated with the culture of orchid protoplasts. This phenomenon was also reported in sugarcane protoplast[22]. Furthermore, raphides released from burst idioblast constitute another factor that causes physical damage to adjacent protoplasts. Factors such as incubation temperature and shaking affect the rate of protoplast release and subsequently the total yield. Elevated temperature of ten hastens wall degradation but since most plant cells deteriorate rapidly at temperatures above 40°C protoplasts used for culture step should not incubate above 30°C[20]. For *Aranda* Chark Kuan the optimum incubation temperature is 30°C which corresponds to that for *Aranda* Tay Swee Eng[10]. Gentle agitation usually aids in isolation of protoplasts and also helps provide adequate aeration. Agitation of *Aranda* Chark Kuan protoplasts was found suitable at 40 rpm. The gentle shaking of the leaf pieces during cellulase treatment causes the loosening of protoplasts from their wall thus increasing the yield. For the nutrient media used, protoplasts had the longest survival in B5 medium. Price and Earle[18] used enriched B5 medium for the study of source of several orchid protoplasts. Such arich medium could be beneficial in replacing anyessential metabolites lost during isolation In general, KC, VW and MS media have been successfully employed in orchid tissue culture and other plant systems. These media failed to support growth of *Aranda* protoplasts although they were supplemented with 2,4-D and BA. Sajise and Sagawa[11] reported the regeneration of plantlets from protoplasts of *Phalaenopsis* sp. cultured in modified VW medium containing 1/4 and 1/2 trace elements of MS medium. They demonstrated that manganese affects the photosynthetic ability of the callus. Furthermore, an optimum concentration of manganese in combination with optimum concentration of each of the other MS microelements has to be established to fully control differentiation of tissues and regeneration of plantlets. The addition of calcium chloride to the isolation medium affected the quality and yield of protoplasts. Shekhawat and Galston [23] reported that the optimum concentration of calcium chloride for moth bean protoplast release was 1 mM; more than 2mM not only inhibited the formation of protoplasts but also caused excessive browning of tissues. The calcium chloride concentration (10 mM) used in this experiment to stabilize membrane is probably too high thus inhibiting fundamental cell processes such as cytoplasmic streaming and mitosis [24].

The early medium of Nagata and Takebe [17], which will initiate cell wall synthesis in protoplasts of tobacco, also succeeds in stimulating wall formation of *Aranda* Chark Kuan leaf protoplasts. In general ammonium is accepted as an important constituent of media for plant tissue culture. However, the monopodial orchid is sensitive to ammonium concentration in the culture medium. Therefore, it is worth noting that ammonium ion was omitted from the NT medium thus providing better wall formation than other tested media. This research is in progress. The most important of the remaining problems is the poor survival of the protoplasts. Attention is now focused on the media formulation to form cell clusters.

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บทคัดย่อ

ตากล้วยไม้อะแรนด้าพันธุ์จักก๊วน ที่เพาะเลี้ยงบนอาหารแข็งสูตร Vacin and Went (1949) เป็นเวลา 1 เดือน สามารถเจริญเป็นโปรโตคอร์มได้ เมื่อฉีกใบที่หุ้มตา พร้อมทั้งตัดยอดออก แล้วย้ายลงเลี้ยงในอาหารเหลวสูตรเดิมโปรโตคอร์มดังกล่าว สามารถพัฒนาเป็นต้นกล้วยไม้ที่สมบูรณ์หลังจากย้ายเลี้ยงบนอาหารแข็งสูตรเดิมอีกครั้งหนึ่ง นำใบอ่อนกล้วยไม้ขนาด 1-3 ซม. ที่ได้จากการเพาะเลี้ยงมาแยกโปรโตพลาสต์ใน สารละลายเอนไซม์ซึ่งประกอบด้วยเซลลูเลส 2% น้ำตาลซอร์บิทอล 0.7โมลาร์ แคลเซียมคลอไรด์และเมสอย่างละ 10 มิลลิโมลาร์ โดยใช้ใบกล้วยไม้น้ำหนักสด 1 กรัม ต่อสารละลายเอนไซม์ปริมาตร 10 มิลลิลิตร นำไปอินคูเบทที่อุณหภูมิ 30 องศาเซลเซียส และเขย่าด้วยความเร็ว 40 รอบต่อนาทีเป็นเวลา 7 ชั่วโมง ได้จำนวนเฉลี่ย 3.6×10^5 โปรโตพลาสต์ต่อมิลลิลิตร ในการเพาะเลี้ยงโปรโตพลาสต์นั้น พบว่า โปรโตพลาสต์ที่เพาะเลี้ยงด้วยจำนวนเริ่มต้น 1×10^5 โปรโตพลาสต์ต่อมิลลิลิตรในอาหารเหลวสูตร Nagata and Takebe (1970) ที่มี 2,4-D(2,4-dichlorophenoxyaceticacid) กับ BA (N^6-benzyladenine) อย่างละ 1 มิลลิกรัมต่อลิตร ในที่มืดเป็นเวลา 3 วัน มีการสร้างผนังเซลล์ใหม่ได้ แต่เมื่อเพาะเลี้ยงต่อไปไม่พบการแบ่งเซลล์