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Isolation and Fusion of Protoplasts from Mesophyll Cells of *Dendrobium* Pompadour

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ABSTRACT Protoplasts were isolated from *Dendrobium* Pompadour leaves grown *in vitro*. Yields of 3x10^5-2.5x10^6 mesophyll protoplasts per gram fresh weight were obtained depending on factors such as digesting enzyme, osmoticum concentration, and leaf size. Small leaves (2.5-3 cm in length and 0.3 g in weight) were digested with 5 ml of enzyme solution containing 1% Cellulase Onozuka R-10, 1% Macerozyme R-10, 0.5% Driselase, and 0.4 M mannitol. The mixture of leaves and enzyme solution was incubated at 30 C for 3 h on a gyr otary shaker with an agitation speed of 80 rpm. Purification of protoplasts on a sucrose gradient yielded very clean protoplasts that are free of raphide crystals. The fusion of protoplasts was carried out using 40% polyethylene glycol. Video microscopic studies revealed that fusion occurred when osmotic gradient was lowered.

KEYWORDS: *Dendrobium*, fusion, protoplast, polyethylene glycol, PEG.

INTRODUCTION

The potential of plant regeneration using protoplasts provides the basis for possible cell selection, somatic cell hybridization and genetic manipulation. To pursue this approach, efficient protocols for isolation and fusion as well as plant regeneration are essential. Protoplast culture for orchids is generally known to be difficult. To date, little research on protoplast culture has been reported in some orchid genera such as *Renantanda,*[1-2] *Dendrobium*,[3] *Aranda*,[4-6] *Phalaenopsis*,[7] *and Cymbidium.*[8] The orchid cut flower industry in Thailand has been a major foreign exchange earner and 92% of the orchids grown for cut flowers are *Dendrobium*.[9] Therefore in this report, we describe our attempts to develop the procedure for isolation and fusion of *Dendrobium* protoplasts. This study is part of the large project designed to explore the possibility to create intergeneric somatic hybrids of orchids by protoplast culture technology.

MATERIALS AND METHODS

Plant material

Dendrobium Pompadour plantlets derived from meristem culture following the methods described by Kanchanapoom and Tongseedam[6] were used in this work.

Nutrient media and culture conditions

Dendrobium plantlets were grown aseptically in 115 ml screw-topped jars each containing 50 ml Murashige and Skoog medium[10] supplemented with 3% (w/v) sucrose. The pH of the medium was adjusted to 5.7 with 0.1 N NaOH or HCl prior to the addition of 0.7% (w/v) Difco Bacto agar. The medium was sterilized by autoclaving at 1.05 kg/cm^2 at 121° C for 20 min. The cultures were incubated at 25-27°C with a 15-h photoperiod, and 20 μmolm^{-2}s^{-1} photosynthetic photon flux density provided by Gro-Lux lamps. The young plantlets were subcultured once a month.

Protoplast isolation and purification

Leaves with the size longer than 2.5 cm was used as source of protoplasts. All leaves were preplasmolysed for 1 h in CPW[11] salts and 0.7 M mannitol in the dark and then were chopped transversely into strips about 1-2 mm in width. Approximately 0.3 g of leaf strips were used per 5 ml of filter-sterilized enzyme solution in 50-mm Pyrex Petri dishes. The enzymes used were 1% Cellulase Onozuka R-10 (Yakult Honsha Co., Ltd. Lot # 201059), 0.5% Driselase (Kyowa Hakko Co., Ltd. Lot # 4111) and 1% Macerozyme R-10 (Yakult Honsha Co., Ltd. Lot # 202021). All enzymes were dissolved in 0.4 M mannitol at pH 5.7 unless otherwise stated. The leaf-enzyme mixture was placed on a rotary shaker (80 rpm; 30°C) in the dark. After 3-h incubation, protoplasts were sieved through a 61 and 43 μm mesh stainless steel screen to remove any clumps of undigested tissues and debris. The filtered protoplasts were centrifuged at 40-xg for 5 min. The filtrate was removed using a Pasteur pipette. The pellet was resuspended and washed twice in 0.4 M mannitol. Finally the pellet material was transferred to the top of a 15-ml screw capped centrifuge tube containing 6 ml of 0.5 M sucrose solution. Centrifugation was performed at 80-xg for 10 min and protoplasts were observed on the surface of the sucrose solution while the remaining cells and debris was sedimented to the bottom of the tube. Protoplasts were gently collected and washed 2-3 times with 0.4 M mannitol.

Assessment of protoplast yield, viability and wall formation

The clean protoplasts were counted using an AO Bright-Line hemacytometer slide at 100x. The viability of freshly isolated protoplasts was monitored using fluorescein diacetate (FDA) as described by Widholm.[12] The presence of wall material was monitored using Calcofluor white (Sigma) as described by Nagata and Takebe.[13] The fluorescence of the labeled protoplasts was determined using an Olympus BH2-RFL microscope as described by Kanchanapoom and Tongseedam.[6] All results were from three independent experiments, three replicates per experiment.

Protoplast fusion

One drop of dense protoplast suspension was pipetted onto the middle of the microscope slide and allowed the protoplasts to settle to the bottom for 5 min. Fusion was induced by adding a drop of 40% polyethylene glycol solution to the settled protoplasts. Treated protoplasts were incubated on the slide for at least 20 min at room temperature. For fusion observation, protoplasts were covered with a cover slip, and the washing solution was perfused across the slide.

Monitoring protoplast fusion events

Fusion was examined using an Olympus inverted microscope IMT-2 equipped with fluorescence attachment connecting with a TV-camera that transmitted the microscopic image to the screen. The fusion of protoplast can be recorded with a color video camera coupled to the inverted microscope optics via a port above the microscope stage. The video camera was connected to a video cassette recorder and color video printer which recorded the experiments and their time course on the VHS color tape and color printing pack, respectively. A 35-mm camera (Olympus SC 35) was coupled to the microscope optics through a second porthole in the base of the microscope for purpose of color photographs (Kodacolor ASA 200 film) at specific instances during the progress of the experiments. The experiments were monitored through a color TV, as well as through the microscope. With this set-up, it is possible to follow and catch up the fusion events.

Results

Protoplast isolation

In order to optimize protoplast isolation, the following parameters were considered: light and dark condition, size of donor plants, type and levels of enzymes, incubation time, and osmolarity. The effect of light seems not to be a critical factor in isolation since young *in vitro* grown plants that were kept in darkness 24h prior to isolation gave protoplast yield which did not result in higher yield than in the case of untreated plants (Table 1). In all the remaining work therefore we directly used without any dark preincubation.

The length of leaf explants between shorter than 2.5 cm and longer than 2.5 cm was compared. As it appeared in Table 2, protoplasts released from leaf of size longer than 2.5 cm gave protoplasts of greater number at 3 h incubation. The protoplasts from longer leaves were larger than shorter leaves. The protoplasts varied in size from 50 to 80 μm in diameter and contained several chloroplasts (Fig 1a). In addition, protoplasts derived from *Dendrobium* mesophyll cells were intact and stable. Many features of chloroplast distribution are common in *Dendrobium* and different orchid taxa such as some protoplasts had chloroplasts distributed throughout the entire cytoplasm (Fig 1b), some had translucent or colorless cytoplasm (Fig 1c), while others had clusters of chloroplasts at one side of the intact protoplast (Fig 1d).

Mesophyll cells of *Dendrobium* like other plant families such as Rubiaceae, Acanthaceae and Malvaceae, which contain idioblast cells (Fig 2a). Raphide crystals (Fig 2a) that had been released from idioblasts during the degradation of cell wall were observed. The deleterious damage effect from these crystals was clearly seen in Fig 2b that the sharpness of needle-like structure could pierce and burst the protoplasts. However, crystals were reduced to

Table 1. Effect of light and dark regime on the release of protoplasts from 1% Cellulase + 1% Macerozyme + 0.5% Driselase in 0.4 M mannitol at various incubation times.

Incubation time (h)	Protoplast yields (x10^5 per g f wt) Light	Dark
1	3.63 ± 0.48*	3.93 ± 0.68
2	13.00 ± 0.41	13.37 ± 1.38
3	25.00 ± 0.24	25.33 ± 1.55
4	22.50 ± 1.84	21.70 ± 0.62

* Values represent the mean number (± standard deviation) of protoplasts harvested on 3 different experiments. Student's t-test shows no significant deference between treatments at the 95% confidence level.

Table 2. Effect of leaf size on the release of *Dendrobium* protoplasts from 1% Cellulase + 1% Macerozyme + 0.5% Driselase in 0.4 M Mannitol.

Incubation time (h)	Protoplast yields (x10^5 per g f wt) < 2.5 cm	> 2.5 cm
1	8.27 ± 1.16*	10.32 ± 3.35
2	14.53 ± 1.54	25.84 ± 3.90
3	28.33 ± 2.86	31.12 ± 3.08
4	30.90 ± 1.07	21.70 ± 1.28

* Values represent the mean number (± standard deviation) of protoplasts harvested on 3 different experiments. Student's t-test shows no significant deference between treatments at the 95% confidence level.

Table 3. Effect of two enzyme mixtures on the isolation of *Dendrobium* mesophyll protoplasts at different incubation times.

Incubation time (h)	Protoplast yields (x10^5 per g f wt) E 1*	E 2
1	0.33 ± 0.12**	1.48 ± 0.54
2	3.49 ± 0.90	10.17 ± 1.43
3	12.73 ± 1.56	22.90 ± 3.49
4	10.10 ± 4.02	21.07 ± 2.29

* E1 = 1% Cellulase + 1% Macerozyme in 0.4 M mannitol at pH 5.6

E2 = 1% Cellulase + 1% Macerozyme + 0.5% Driselase in 0.4 M mannitol at pH 5.6

** Values represent the mean number (±standard deviation) of protoplasts harvested on 3 different experiments. Student's t-test shows significant deference between treatments at the 95% confidence level.

Table 4. *Dendrobium* mesophyll protoplasts released from 1% Cellulase + 1% Macerozyme + 0.5% Driselase at various mannitol concentrations.

Incubation time (h)	Protoplast yields (x10^5 per g f wt) 0.4	0.5	0.6 (M)
1	4.45 ±1.90**	1.19 ±0.81	2.25 ±2.52
2	11.56 ±4.73	5.49 ±3.34	5.76 ±4.71
3	19.89 ±4.98	13.59 ±4.32	6.95 ±3.04
4	19.53 ±3.61	15.86 ±4.56	12.46 ±3.82

**Values represent the mean number (± standard deviation) of protoplasts harvested on 3 different experiments. Student's t-test shows significant deference between treatments at the 95% confidence level.

the minimum by the sucrose floatation and sedimentation protocol. Therefore the isolation and purification techniques described here yielded very clean populations of protoplasts.

The type of enzymes and their concentrations affected the number of viable protoplasts. To determine the optimum time of cell wall digesting enzyme action for obtaining high yield and good quality of protoplasts, two enzyme mixtures were used to test the release of protoplasts as shown in Table 3. It was found that 22x10^5 protoplasts per gram fresh weight (g.f.wt.) of leaf were achieved with a digestion period of 3 h when Driselase was included in the enzyme solution. The freshly isolated protoplasts were of spherical shape and were yellow-green with FDA fluorescence and red with chlorophyll fluorescence indicating viability after release. Calcofluor white staining indicated that cell wall removal was complete after isolation. Therefore we routinely used 1% Cellulase + 1% Macerozyme + 0.5% Driselase for protoplast isolation.

The osmolarity of the enzyme solution had a substantial effect on the yield and viability of the protoplasts. In this work several concentrations of mannitol as well as sorbitol were used in the enzyme incubation medium and washing medium. Of the range of concentrations tested (0.4-0.6 M), the highest number of protoplasts obtained was 19.89x10^5 per g.f.wt. at 3 h in 0.4 M mannitol (Table 4). This suggested that the distinct optimum concentration of osmoticum suitable for protoplast releasing was 0.4 M.

Protoplast fusion

On addition of PEG the protoplasts were instantly shocked and lost their round shape. The monitoring of fusion events after the washing solution was perfused across the slide using video microscopy as shown in Fig 3 revealed that two protoplasts came into close contact with each other (Fig 3a). The protoplast membrane fused, and a connection was formed between the two cytoplasm (Fig 3b). Finally, the two protoplasts formed a spherical fusion product (Fig 3c). However, a fusion frequency of about 14% was obtained with this method.

Discussion

The aim of this research was to develop techniques for the fusion of orchid protoplasts to facilitate somatic hybridization. Prerequisite for the practical use of protoplasts is efficient methods for isolation. The successful isolation of protoplasts depends on

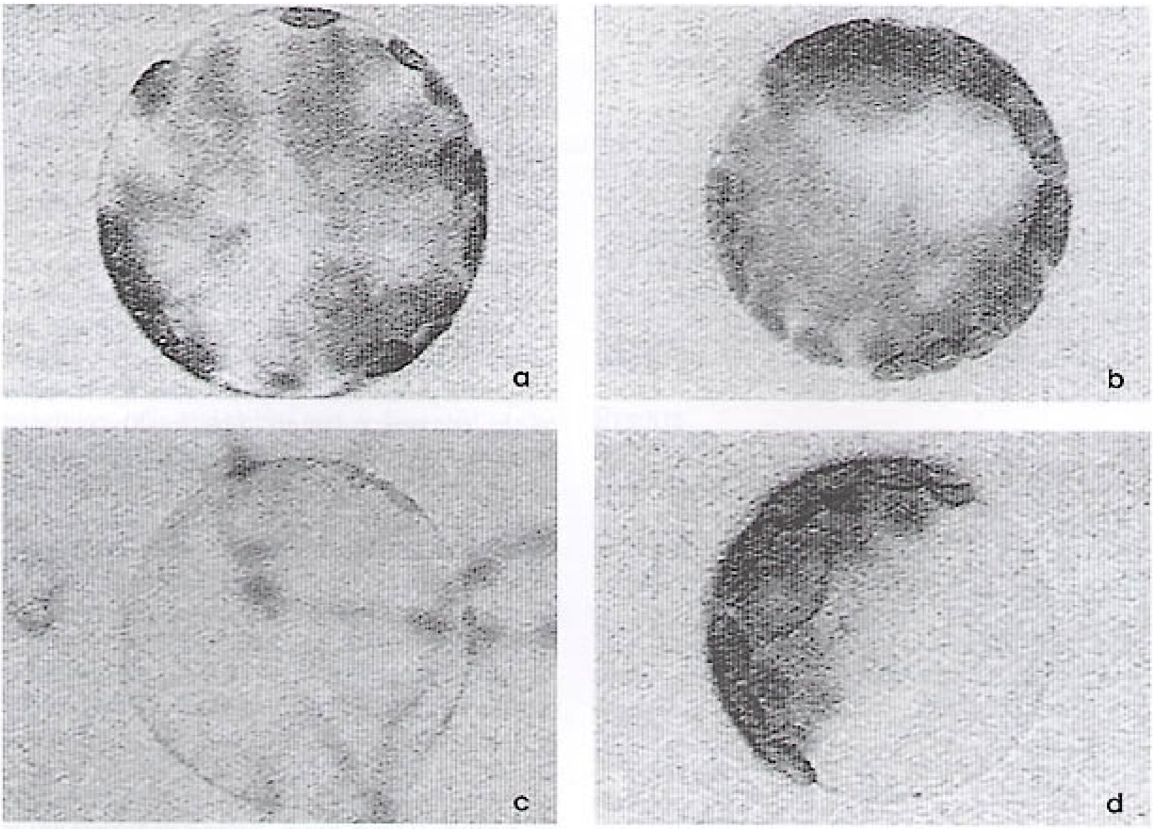

Fig 1. (a) Freshly isolated protoplasts of *Dendrobium*. (b) Chloroplasts arranged randomly. (c) Translucent protoplast without chloroplasts. (d) Aggregation of chloroplasts at one side.

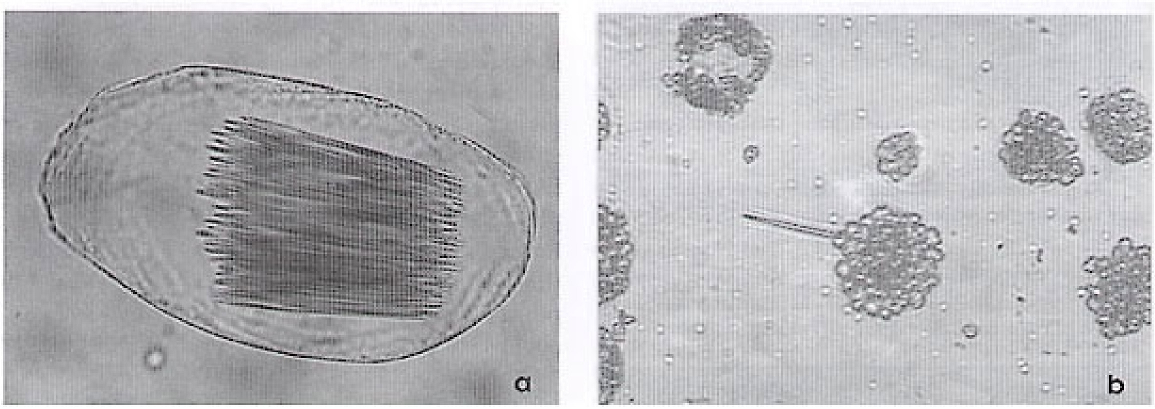

Fig 2. (a) Raphide crystals arranged orderly within an idioblast cell. (b) A free crystal was piercing a protoplast resulting in death of protoplast.

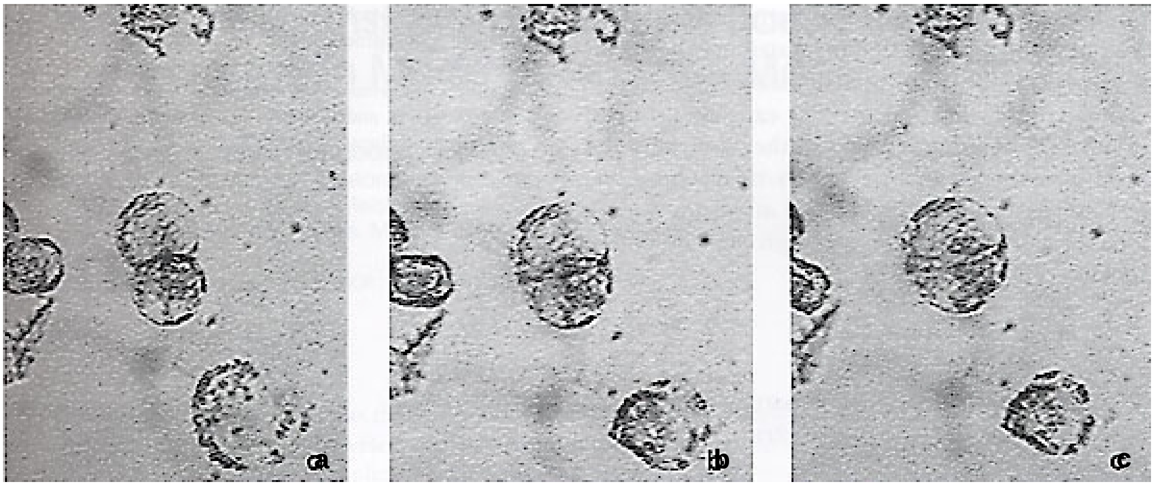

Fig 3. Video image of *Dendrobium* mesophyll protoplasts induced to fuse by 40% PEG solution. (a) A few seconds after initial contact of two protoplasts. (b) Lipid bilayer became fused, and the cytoplasm between two adjacent protoplasts was connected. (c) The spherical fusion product was formed.

many factors that are related to the source plant and the conditions of the applied methods. The isolation conditions are extremely important for the release of protoplasts without impairing cell viability and to achieve maximum yield. *In vitro* grown *Dendrobium* which was kept in darkness, gave protoplast yield that did not differ from those cultured in light suggested that both plants might have the same metabolic state. Size of leaves was an important factor affecting yield and quality of protoplasts both in other plants such as grapevine[14] and *Aranda* Tay Swee Eng.[5] In *Dendrobium* leaf explants with the length shorter than 2.5 cm and longer than 2.5 cm did not give significantly different protoplast yield at 95 % level. On the other hand, protoplasts from longer leaves were larger and prone to further manipulations. However, older and longer leaves contained numerous crystal, presumably calcium oxalate, may deflate and kill protoplasts. Oshiro and Steinhart[8] stated that orchids stored some of their photosynthetic harvest in the form of oxalic acid, an oxidation product of intermediates of the glycolic acid metabolic pathway. Oxalic acid in a cell and calcium oxalate formed crystals called raphides. The sucrose floatation and sedimentation procedure employed in our method provided a clean protoplast suspension from raphides. The distribution of chloroplasts was widely observed in both orchids and other plant genera.

Among the hydrolytic enzymes used in this experiment, Cellulase Onozuka R-10 and Macerozyme R-10 were found to be effective in releasing protoplasts from the leaves. A reproducible high yield of protoplasts was obtained when the enzyme Driselase was included in the enzyme mixture. The addition of Driselase in the enzyme mixture favored cell wall digestion. This is in agreement with the findings of Loh and Rao[4]; Koh *et al* [5] who reported that inclusion of 0.5% Driselase into the enzyme mixture of 1.5% Cellulase Onozuka R-10 and 0.5% Macerozyme R-10 increased the yield of *Aranda* hybrids mesophyll protoplasts. Furthermore optimum duration of enzyme action was determined to be 3 h, which is lower than that applied by Koh *et al*.[5] Therefore the use of Driselase seemed to be essential to obtain a high yield of protoplasts. However, for the culture manipulations, the use of Driselase must be taken into consideration since inclusion of Driselase was found to be particular harmful for subsequent division of the isolated *Pithecellobium dulce* protoplasts.[15]

The concentration of mannitol used was found to be effective since lysis of protoplasts was not evident. The sphericity of protoplasts could be the result of optimal medium used for the isolation of the protoplasts. In contrast to the work of Loh and Rao;[4] Koh *et al*[5] who employed 0.4 M sucrose as an osmoticum in the enzyme mixture. The use of sucrose in the wash medium was found to be effective in separating protoplasts from debris in their systems.

A high initial aggregation frequency was obtained if protoplasts were used immediately after isolation. With video microscopy, we were able to illustrate true fusion of orchid protoplasts. Osmotic gradient has been shown conclusively by this research to be the driving force for membrane fusion. While protoplasts adhered in the presence of PEG, very little fusion occurred as observed by light microscopy until PEG was diluted and osmolarity lowered. This phenomenon was also demonstrated in other plant protoplasts.[16, 17] This would indicate that osmotic changes upon dilution of PEG caused fusion to occur. FDA staining recognized the survival of the protoplasts after routine fusion treatment. This research is in progress and our attention is now focused on the culture of fusion products to obtain somatic cell hybrids.

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