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# Protoplast Isolation and Culture of *Dendrobium* Sonia "Bom 17"

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## ABSTRACT

The optimization of *Dendrobium* Sonia "Bom 17" protoplast isolation and culture conditions were investigated. Protoplasts were successfully isolated from one-month-old leaves of *in vitro*-grown plantlets using an enzyme solution comprising 1% Cellulase Onozuka R-10, 0.2% Macerozyme, 0.3 M mannitol, 10 mM $CaCl_2.2H_2O$ and 10 mM 2 (*N*-morpholino)-ethanesulfonic acid (MES) at pH 5.8. Approximately $3.97\pm0.63 \times 10^5$ protoplasts per gram fresh weight were obtained when digested in enzyme solution for 4 h on a shaker under dark conditions, then washed with 0.3 M mannitol solution supplemented with 10 mM $CaCl_2.2H_2O$ and 10 mM MES, and purified with 0.3 M sucrose solution. Freshly isolated protoplasts were then cultured at final density of $2 \times 10^5$ protoplasts/ml in Kao & Michayluk medium (KM8P) by agarose-bead cultures under dark conditions at 25°C. First cell division was observed after culturing for 2 days and multicellular colonies (15-20 cells) were formed after 2 weeks.

**Key words:** protoplasts, isolation, culture, *Dendrobium*, microcolony

## INTRODUCTION

Orchid plants are members of Orchidaceae, consisting of more than 800 genera and 25,000 species. They are distributed throughout most of the world, but are more abundantly found in the tropics (Arditti, 1992). There are 177 genera, 1,125 species of orchids native to Thailand (Nanakorn and Indhamusika, 2000). Cut flower orchid export was established in 1968. Since then, Thai orchids have been listed among the top ranked in the world orchid markets. *Dendrobium* and its hybrids are the most important cut flowers and pot orchids for export from Thailand. The significant characteristics of *Dendrobium* flowers are their different colors and forms, long vase life and fragrance in some species. According to the market demand, Thai orchid farmers must produce new varieties with new colors and/or forms. This is a way to boost the demand of Thai orchids.

Many outstanding *Dendrobium* cultivars and hybrids came from conventional methods and somaclonal variation resulting from tissue culture techniques. Although there are many intrageneric and interspecific *Dendrobium* hybrids, it is difficult to produce the intergeneric hybrids using a conventional breeding technique. Somatic hybridization through protoplast fusion allows the combination and hybridization of different

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sexually incompatible genera and species (Davey *et al*., 2005). Hence this technique may provide opportunities for hybridization of *Dendrobium* with other orchid genera. To achieve success in application of using protoplasts as targets of somatic hybridization and direct gene transfer for improvement of new *Dendrobium* hybrids, the isolation of the viable and cultureable orchid protoplasts in large quantities is one of the critically important factors. Many factors affect the yield of viable protoplasts, such as material source, age of plants, enzyme mixture, isolation medium, concentration of osmoticum solution and incubation time. Culture medium and growth regulators, plating density, method of culture and conditions of incubation affect protoplast division (Bhojwani and Razdan, 1983). To date, there are few reports on the isolation and culture of protoplasts from *Dendrobium* cultivars (Kunasakdakul and Smitamana, 2003) and species (Yasugi, 1989). However, standardized methods are adopted according to the species and variety of the plant. Therefore, this study would emphasize the optimization of isolation and culture techniques of *D*endrobium Sonia "Bom 17" protoplasts to provide basic research data on *Dendrobium* improvement.

## MATERIALS AND METHODS

### 1. Plant materials

Protocorm-like bodies (PLBs) of *Dendrobium* Sonia "Bom 17" were obtained from Hatai Tissue Culture Laboratory, Pathum Thani Province. The PLBs were subcultured on modified Vacin and Went (VW) medium (Vacin and Went, 1949) supplemented with 15% coconut water, 10% banana homogenate, 5% mashed potato, 0.2% activated charcoal, 2% sucrose and 0.8% agar at pH 4.8. The cultures were incubated under a 16/8 h light/dark photoperiod at 25±2°C. Leaves of plantlets regenerated from PLBs were used as the explants for protoplast isolation.

### 2. Optimal conditions for protoplast isolation

#### 2.1 Enzyme solution

Three different kinds of enzyme solutions were examined for the best protoplast isolation. The tested enzyme solutions were as follow: E1, 0.5% Cellulase Onozuka R-10 (Phyto Technology Laboratories, USA) and 0.2% Macerozyme (Phyto Technology Laboratories, USA); E2, 1% Cellulase Onozuka R-10 and 0.2% Macerozyme; and E3, 1.5% Cellulase Onozuka R-10 and 0.2% Macerozyme. The enzyme solutions were dissolved in the washing solution (0.3 M mannitol, 10 mM $CaCl_2.2H_2O$ and 10 mM MES, pH 5.8). One gram of young leaves was taken from one-month-old *in vitro* orchid plantlets. Leaves were cut transversely into small pieces about 2 mm wide with a razor blade in a 6 cm Petri dish containing 5 ml of washing solution. The sliced tissues were plasmolysed by immersion for 15 minutes after that the washing solution were replaced with 5 ml of the filter-sterilized (Satorius, pore size 0.20 mm) enzyme solution. The Petri dishes were sealed with parafilm and incubated in the dark on a gyratory shaker set at 40 rpm for 4 h. After digestion, the incubation mixture was then gently filtered through a 60 mm nylon mesh to remove undigested tissue and debris. The filtrate was centrifuged for 5 min at 750 rpm in a swinging bucket centrifuge (Kokusan, Japan). The supernatant was discarded and the protoplast pellet was resuspended in the washing solution and centrifuged for 3 min at 750 rpm. This process was repeated one more time. The protoplasts were purified by floating on 0.3 M sucrose solution and centrifuged at 850 rpm for 10 min. The protoplasts floating at the interface between the sucrose and mannitol solutions were collected. The purified protoplasts were then washed twice with washing solution by centrifugation at 750 rpm for 3 min each. The purified protoplasts were resuspended in 1 ml of the washing solution. Protoplast yield was counted using a Boeco haemocytometer (Germany) under an microscope (Nikon ECLIPSE TE 300, Japan). Protoplast viability was examined

by staining with 0.01 % (W/V) fluoresceine diacetate (FDA) in 0.3 M mannitol under a fluorescence microscope (Nikon ECLIPSE TE 300, Japan) (Larkin, 1976).

**2.2 Age of leaves**

One gram of one or two-month-old leaves was used for protoplast isolation with an appropriate enzyme solution from the experiment 2.1. Protoplast yield and viability were determined as in 2.1.

**2.3 Incubation period**

One gram of one-month-old *in vitro* leaves was incubated in 5 ml of an appropriate enzyme solution from the experiment 2.1. The incubation was carried out in the dark on a shaker set at 40 rpm for 3, 4, 5 or 6 hours. The protoplasts were harvested and purified as previously described in 2.1. Protoplast yield and viability were determined.

**2.4 Concentration of osmoticum solution**

One gram of one-month-old *in vitro* leaves was incubated in 5 ml of an appropriate enzyme solution and the protoplasts were isolated and purified as previously described in 2.1. The protoplasts were washed with 0.2, 0.3, 0.4 or 0.5 M mannitol solutions. Protoplast yield and viability were determined.

**3. Optimal conditions for protoplast culture**

**3.1 Protoplast density**

Purified protoplast at densities of $0.5 \times 10^5$, $2 \times 10^5$ and $5 \times 10^5$ protoplasts/ml were cultured with the agarose bead method. One volume of the protoplast suspension was gently mixed with one volume of KM8P medium (Kao and Michayluk, 1975) containing 0.6% (w/v) molten agarose Seaplaque (FMC, BioProducts, Rockwell, U.S.A.). The protoplast suspension was pipetted and dropped into a 6 cm Petri dish. After the gelling of agarose, the droplets were covered with 3 ml of KM8P medium. Petri dishes were sealed with parafilm and incubated at 25 °C in the dark. The percentage of dividing protoplasts was examined after 1 week of culture.

**3.2 Culture medium**

Protoplasts at a density of $2 \times 10^5$ protoplasts/ml were cultured in two kinds of culture media; KM8P or modified MS (Yasugi, 1989) using the agarose bead method as previously described in 3.1.

**3.3 Culture method**

Protoplasts were cultured using two methods, the liquid thin layer and agarose bead methods. For the liquid thin layer method, 2.5 ml of the purified protoplasts at density of $2 \times 10^5$ protoplasts/ml in liquid KM8P medium were poured into a 6 cm Petri dish. For the agarose bead method, the protoplasts were cultured as described above in 3.1. The Petri dishes were sealed with parafilm and incubated at 25 °C in the dark. The cell division was observed periodically with an inverted microscope (Nikon ECLIPSE TE 300, Japan). The initial plating efficiency (% of plated protoplasts under cell division) was determined after 1 week of culture. The final plating efficiency (% of protoplasts that underwent cell division to form microcolonies of 10-20 cells was determined after 2 weeks of culture.

**4. Data analysis**

All experiments had three replicates. The data were assessed by one-way analysis of variance (ANOVA) and the means were compared by the Tukey test at 95% confidence of interval ($p<0.05$). The significance of difference in protoplast division as influenced by the protoplast density and culture media were assessed by independent sample t-test. All the statistical analyses were carried out using SPSS 11.0 software (SPSS, Chicago, IL, USA).

## RESULTS AND DISCUSSION

**1. Optimal conditions for protoplast isolation**

**1.1 Enzyme solution**

Protoplasts could be isolated with all enzyme solutions. Among the three enzyme

solutions tested, the highest yield of 5.33± 1.12 × $10^5$ protoplasts/gram fresh weight was obtained from the leaf digested with the enzyme mixture of 1.5% Cellulase Onozuka R-10 and 0.2% Macerozyme (E3). However, it was not significantly different from those of E1 (3.23±0.93 × $10^5$ protoplasts/ gram fresh weight) and E2 (3.97±0.63 × $10^5$ protoplasts/ gram fresh weight) at $p>0.05$ (Figure 1). The viability of protoplasts (95.61±1.24%) of E2 was higher than E3 (92.61±1.24%) (Figure 1). According to Asano (1993), a high enzyme level was deleterious to plant cells, therefore, the protoplasts isolated in a low enzyme level showed a better growth response. The suitable enzyme solution for *Dendrobium* Sonia "Bom 17" protoplast isolation was E2.

**1.2 Age of leaves**

Protoplasts could be isolated from both ages of leaves. There was significant difference between the viability of protoplast but not significant difference in the yield ($p>0.05$) (Figure 2). However, more protoplasts per gram fresh weight were obtained from two-month-old leaves than one-month-old leaves (Figure 2). Similarly, Koh (1992) found that older leaves (1-3 cm length) gave higher yields compared to younger leaves (< 1 cm length). The age of leaves affected the yield of protoplasts. There were many crystals, the, raphides when using two-month-old leaves as the protoplast source (Figure 5B). This might result from older leaves accumulating more metabolic products than younger leaves. These raphides have been found in orchid cells. They are formed by oxalic acid and calcium oxalate in orchid cells (Oshiro and Steinhart, 1991). These crystals can puncture and burst the protoplasts during isolation (Kunasakdakul and Smitamana, 2003). However, all raphides could be successfully removed by purifying protoplasts with 0.3 M sucrose. The procedure of purification of protoplasts established in this study was similar to that developed for isolation of protoplasts from other *Dendrobium* cultivars (Kunasakdakul and Smitamana, 2003), by using 0.3 M (10%) sucrose instead of 20% sucrose. Generally, protoplasts with high viability have potential to redifferentiate after dedifferentiation in culture (Li and Chen, 1983). Therefore, a one-month-old leaf (Figure 5A) was the appropriate age for protoplast isolation of *Dendrobium* "Bom 17". Protoplasts obtained from

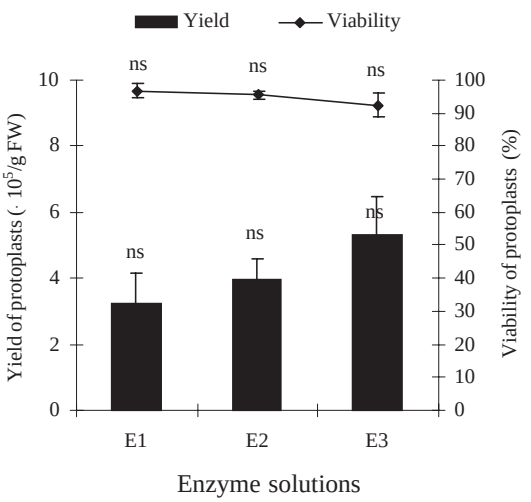


**Figure 1** Effect of different enzyme solutions on yield and viability of *Dendrobium* Sonia "Bom 17"protoplasts. Error bars represent SE.

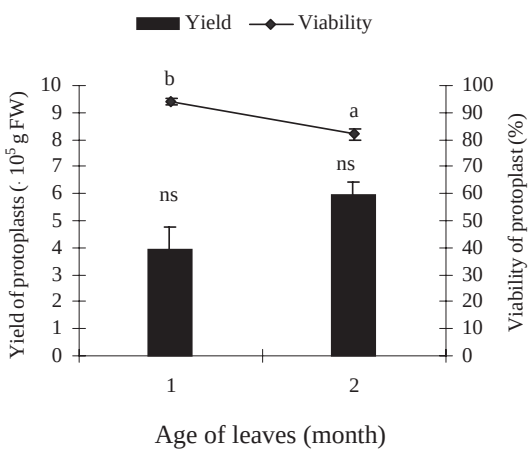


**Figure 2** Effect of leave age on yield and viability of *Dendrobium* Sonia "Bom 17" protoplasts. Error bars represent SE.

one-month-old leaves are shown in Figure 5C. Its viability was 94.17±1.18 % as determined by FDA staining (Figure 5D).

**1.3 Incubation time**

Among the incubation times tested, the highest yield 4.80±1.84 × $10^5$ protoplasts/gram fresh weight with 89.77±5.81% viability was obtained when the leaves were incubated in enzyme solution for 5 h. It was not significantly different from protoplasts in those of 3, 4 and 6h at p>0.05 (Figure 3). When the incubation time was 6 h, the yield and viability of protoplasts decreased. Similarly, Shrestha (2003) reported that most protoplasts derived from the callus of *Phalaenopsis* "Wataboushi" were disrupted after being incubated in the enzyme solution for 24 h. Therefore, the optimal incubation time for isolation of *Dendrobium* Sonia "Bom 17" protoplasts was 4 h which gave a higher viability of protoplasts (90.79±5.93%). Usually, isolation of *Dendrobium* protoplasts is performed for a period of 3-16 hours in the dark (Kanchanapoom *et al.*, 2001; Kunasakdakul and Smitamana, 2003).

**1.4 Concentration of osmoticum solution**

An appropriate concentration of mannitol solution as an osmoticum for protoplast isolation was determined. The results showed that concentration of mannitol solution significantly influenced yield and viability of protoplast (p<0.05). The 0.3 M mannitol solution produced the highest yield (2.79±0.45 × $10^5$ protoplasts/ gram fresh weight) and highest viability of protoplasts (97.19±0.03%) (p<0.05) (Figure 4). Mannitol solution at 0.5 M completely reduced yield and viability of protoplasts since all protoplasts were plasmolyzed. On the other hand, some protoplasts in 0.2 M mannitol solution were swollen and disrupted. The results indicated that the mannitol concentration was another crucial factor for protoplast isolation. Mannitol at a concentration of 0.3–0.6 M was used in *Dendrobium* protoplast isolation (Sajise *et al*., 1990; Hu *et al*., 1998).

**2. Optimal condition for protoplast culture**

**2.1 Protoplast density**

The purified protoplasts at a density of 0.5 × $10^5$, 2 × $10^5$ and 5 × $10^5$ protoplasts/ml were cultured in KM8P medium with agarose bead method. Protoplasts density significantly affected

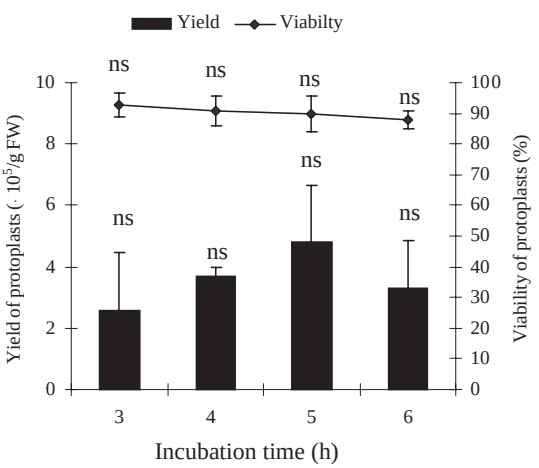


**Figure 3** Effect of incubation time on yield and viability of *Dendrobium* Sonia "Bom 17" protoplasts. Error bars represent SE.

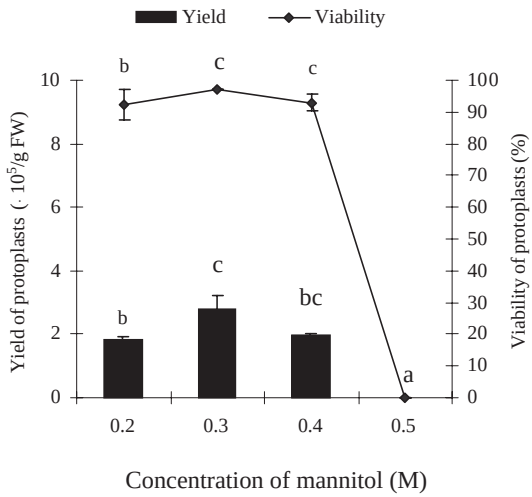


**Figure 4** Effect of mannitol concentration on yield and viability of *Dendrobium* Sonia "Bom 17" protoplasts. Error bars represent SE.

protoplast division ($p<0.05$) (Table 1). The highest cell division (20.18±0.25%) was obtained when cultured at $2 \times 10^5$ protoplasts/ml (Table 1). Protoplasts cultured at a higher density ($5 \times 10^5$ protoplasts/ml) had lower protoplast division (6.45±1.32%). At the lower plating density ($0.5 \times 10^5$ protoplasts/ml) no protoplast division occurred. Therefore, the optimal plating density of *Dendrobium* Sonia "Bom 17" was $2 \times 10^5$ protoplasts/ml, which was higher than those of *Dendrobium noblie* ($5.5\text{-}8.2 \times 10^4$ protoplasts/ml) (Yasugi, 1989) and Hawaiian *Dendrobium* cultivars ($0.5\text{-}0.7 \times 10^5$ protoplasts/ml) (Kuehnle and Nan, 1990). The final or overall density of protoplasts in culture medium (plating density) was crucial for minimizing cell wall and regeneration and concomitant daughter cell formation. An excessively high plating density rapidly depletes nutrients, and protoplast-derived cells can fail to undergo sustained division. Moreover, protoplasts fail to undergo sustained division when cultured below a minimum protoplast density (Davey *et al*., 2005). It is thought that cell leakage into the medium enriches the medium to sustain cell growth and that, if the protoplast density is too low, too few endogenous chemical will leak into the medium (George and Sherrington, 1984).

**2.2 Culture medium**

The effect of culture medium on protoplast division was investigated using KM8P and modified MS medium. Significant differences in protoplast division was found ($p<0.05$) (Table 2). Protoplasts cultured in modified MS medium did not show any cell division (Table 2). The protoplasts remained intact for 3 days and were then turned brown, while all protoplasts cultured in KM8P medium changed their size after 24 h. The first cell division of protoplasts occurred after 2 days of culture. The percentage of protoplast division was 22±0.23 after 7 days of cultur. It was indicated that KM8P medium was suitable for culturing mesophyll protoplasts of *Dendrobium* Sonia "Bom 17". The KM8P medium was an effective medium since it was enriched with vitamins, organic acids, sugar, casamino acids and coconut water and it was found to be very effective for protoplasts of recalcitrant species (Tamura *et al*., 1995). The media that have been used successfully for *Dendrobium* protoplast cultures are modified PS (Kunasakdakul and Smitamana, 2003) and modified MS medium (Kuehnle and Nan, 1990).

**2.3 Culture method**

The protoplasts were cultured using two methods; liquid culture and agarose bead culture. The culture methods significantly affected the initial and final plating efficiencies of *Dendrobium* Sonia "Bom 17" protoplast. The protoplasts cultured in the liquid thin layer started dividing after a few days. The initial plating efficiency was 5.45±1.05 % by the 7$^{th}$ day (Table 3). Protoplasts underwent budding at the 8-10$^{th}$ day, caused by incomplete cell wall regeneration (Meyer and

**Table 1** Effect of protoplast density on protoplast division of *Dendrobium* Sonia "Bom 17".

| Protoplast density (protoplasts/ml) | Protoplast division (%) |
|---|---|
| $0.5\times10^5$ | $0^a$ |
| $2\times10^5$ | $20.18\pm0.25^c$ |
| $5\times10^5$ | $6.45\pm1.32^b$ |

Means in the same column not sharing the common superscript letter are significantly different determined by t-test. Error bars represent SE.

**Table 2** Effect of the culture medium on cell division of *Dendrobium* Sonia "Bom 17" protoplasts.

| Media | Protoplast division (%) |
|---|---|
| MS | $0^a$ |
| KM8P | $22\pm0.23^b$ |

Means in the same column not sharing the common superscript letter are significantly different determined by t-test. Error bars represent SE.

**Table 3** The initial and final plating efficiencies of *Dendrobium* Sonia "Bom 17" protoplast culture.

| Culture method | Initial plating efficiency (%) | Final plating efficiency (%) |
|---|---|---|
| Liquid culture | 5.45±1.05[a] | 0[a] |
| Agarose bead culture | 17.91±3.53[b] | 4±1.05[b] |

Means in the same column not sharing the common superscript letter are significantly different determined by t-test. Error bars represent SE.

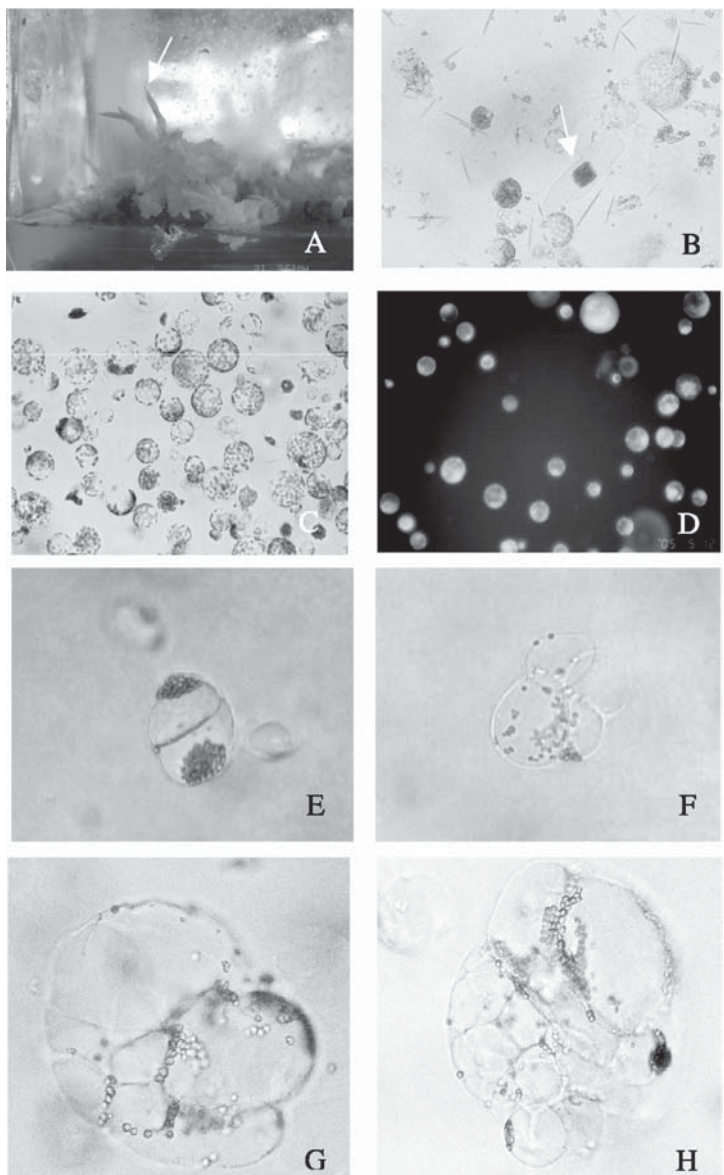


**Figure 5** Isolation, culture and cell division of *Dendrobium* Sonia "Bom 17" protoplasts. One-month-old orchid plantlets suitable for the isolation of leaf protoplasts (arrow) (A), raphids (arrow) (B), protoplasts immediately from purification (C), vigorous protoplasts fluorescing a yellow-green color when stained with FDA (D), first cell division after a few days of culture (E), small cell clusters after 4 (F) and 8 days (G) and a protoplast-derived microcolony after 2 weeks of culture (H).

Abel, 1975). Many pearl-chain-like growing cells were observed. By the 20th day, most protoplasts became brown and aggregated. Liquid culture has been used for microcolony induction in Hawaiian *Dendrobium* cultivars (Kuehnle and Nan, 1990) and *Dendrobium* Prathum Red (Kunasakdakul and Smitamana, 2003). However, these results showed that it was not effective for *Dendrobium* Sonia "Bom 17" protoplasts.

While the protoplasts cultured in agarose bead culture, the first cell division occurred after a few days of culture (Figure 5E). The sustained division and colony formation was visible after 2 weeks of culture (Figure 5F, 5G, 5H). The protoplasts ceased dividing and turned brown after 3 weeks, probably because a toxic polyphenolic substance, which was secreted from developing cells, inhibited protoplast growth. In monocots, including orchids, it is very difficult to culture protoplasts isolated directly from leaves (Kunasakdakul and Smitamana, 2003). Plant regeneration has been possible when callus and cell suspension are used as the source of protoplasts isolation and culture in *Phalaenopsis* (Shrestha, 2003).

It was indicated that agarose bead culture was more favorable for *Dendrobium* Sonia "Bom 17" protoplasts compared to thin liquid layer culture. The enhanced protoplast division observed in bead culture was due to the dilution of the inhibitory substances, which were secreted from the cell to the medium (Mizuhiro *et al*., 2001).

## CONCLUSION

In this study microcolony formation was obtained from protoplasts of one-month-old *Dendrobium* Sonia "Bom 17" leaves. Concentration of mannitol solution proved to influence protoplast yield and viability. Culture medium, plating density and method of culture affected protoplast division. Although the optimal conditions for successful isolating and culturing protoplasts of *Dendrobium* Sonia "Bom 17" were reported, further intensive study would still be required for callus proliferation and plantlets regeneration.

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