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Full Length Research Paper

Optimisation of protoplast isolation protocols using *in vitro* leaves of *Dendrobium crumenatum* (pigeon orchid)

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Protoplast isolation from *in vitro* leaves of *Dendrobium crumenatum* was carried out. Factors affecting protoplast isolation, sorbitol concentration, enzyme combinations and concentration and incubation time for isolation were studied. Results obtained showed that 0.5 M sorbitol was the most effective concentration to isolate the protoplast (25.62×10^4 protoplasts/g FW). High yield of protoplasts, 20.40×10^4 protoplasts/ g FW, could also be obtained by using combination of cellulase and pectinase instead of using the enzyme individually. In addition, efficacy of protoplast isolation was improved by 20.12×10^4 protoplasts/g FW, when 2% (w/v) cellulase and pectinase were used; while incubation for 4 h during the isolation process recorded highest protoplast yield, 28.66×10^4 protoplasts/gFW. Based on the results obtained, we suggest that protoplasts isolation of *D. crumenatum* from *in vitro* leaves could be achieved using 0.5 M sorbitol with combination of 2% (w/v) cellulase and pectinase for both enzymes and 4 h incubation time.

Key words: *Dendrobium crumenatum*, cellulase, pectinase, protoplast, sorbitol.

INTRODUCTION

Dendrobium crumenatum is a tropical epiphytic orchid normally found growing on trees in the South East Asia countries. *D. crumenatum* plants growing in the same area can flower simultaneously depending on the change of the temperature in the area. It has very unique attractive whitish fragrant flower that looks like a pigeon. Therefore, *D. crumenatum* is also known as pigeon orchids. However, the flowers have very short shelf-life and last only for two days (Beaman et al., 2001).

D. crumenatum has low commercial value being cut flower or pot plant due to its short flower shelf-life, even though it is easy to grow. Therefore, prolonging the flower shelf-life of this orchid is an important area of study for many physiologists, developmental biologists, molecular geneticists as well as plant breeders. Hence, the isolation of protoplasts for further downstream studies such as protoplast fusion and genetic manipulation to improve the shelf-life of *D. crumenatum* is particularly essential for improving its economical value.

The development of protoplast technology has been given considerable attention and has attained significant progress. Genetic manipulation through protoplast technologies like somatic hybridisation, cybridisation or direct gene transfer can be exploited for plant improvement if a reliable and efficient plant regeneration system from isolated protoplasts could be developed (Papadakis and Roubelakis-Angelakis, 2002). The potential of plant regeneration using protoplasts provides the basis for possible cell selection, somatic cell hybridisation 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, very few researches on orchid (*Renantanda, Dendrobium*, *Aranda*, *Phalaenopsis*, and *Cymbidium*) protoplast study were reported (Teo and Neumann, 1978; Price and Earle, 1984; Koh et al., 1988; Sajise and Sagawa, 1991; Oshiro

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Abbreviations: MS, Murashige and Skoog's medium.

and Steinhart, 1991). In fact, there is no report on the protoplasts isolation and culture of *D. crumunetum.* Therefore, this present study was attempted to study various factors affecting protoplast isolation and thus to develop an efficient protocol for *D. crumunetum* protoplasts isolation.

MATERIALS AND METHODS

Plant materials and medium preparation

The *in vitro* plantlets of *D. crumenatum* were used. They were maintained and multiplied on the half-strength Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962). The medium consists of MS salts and vitamins with 3% (w/v) sucrose. The pH of the medium was adjusted to 5.8±3 and 0.8% (w/v); and agar was used.

All plant cultures were maintained at 25 ± 1°C and 16 h light condition.

Protoplast isolation solution

Standard concentration of digesting enzymes and sorbitol were used for all the protoplast isolation procedures unless specified. The protoplast isolation solution consists of 2% (w/v) cellulase Onozuka R-10 (Duchefa, Netherland), 2% (w/v) pectinase (Sigma, USA), 0.5 M sorbitol and half-strength MS medium.

Standard protocol of protoplast isolation

The protoplast isolation procedures were carried out under aseptic conditions inside the laminar airflow hood whenever necessary. In vitro leaves of *D. crumenatum* were excised and 0.05 g leaf sample was ground. Then, the protoplast isolation solution (500 µL) was added to the explants. The mixture was then incubated for three hours. It was carried out under the light condition and the mixture was agitated at 90 rpm. This incubation time was used for all the protoplast isolation procedures unless specified. After incubation, the enzyme digested-tissues in the protoplast isolation solution were centrifuged at 5000 rpm for 3 min. The supernatant was collected after centrifugation and the pellet was resuspended using 100 µL of the isolation solution. The process of centrifugation and collecting the resuspension was repeated three times. Lastly, the collected resuspended solution was observed under the stereo microscope and the number of protoplasts obtained was counted using a haemocytometer. All experiments were carried out in triplicate and were repeated.

Effects of different concentrations of sorbitol on protoplast isolation

In this study, sorbitol acts as the sole osmoticum. To identify the optimal concentration of sorbitol that influences the yield of protoplast, 0.5 and 1.0 M sorbitol was used for protoplast isolation and the control protoplast isolation solution used was without sorbitol.

Effects of enzymes on protoplast isolation

To determine the suitable conditions of enzymes used for protoplast isolation, different enzyme combinations and concentrations were used. The effects of applying a single enzyme (cellulase or pectinase) and the combination of both enzymes for protoplast isolation were studied. For this study, 2% (w/v) cellulase and pectinase individually or a combination of 2% (w/v) of both the enzymes were used in the protoplast isolation process.

In addition, the effect of different concentration combinations of cellulase and pectinase was also studied for protoplast isolation. The combinations of 1% (w/v) cellulose, 1% (w/v) pectinase, 2% (w/v) cellulase and 2% (w/v) pectinase were used to investigate their efficacy for protoplast isolation, and protoplast isolation solution without enzyme was used as the control.

Effects of incubation time on protoplast isolation

Incubation time is the duration required to immerse the minced leaf samples in the protoplast isolation solution. To determine the suitable duration required for obtaining the highest yield of protoplasts, the excised and minced leaf samples were incubated with enzymes for different incubation times (0, 2, 4 and 6 h).

Determination of the yield of protoplasts

The isolated protoplasts were observed under the microscope and the number of protoplasts isolated was counted using a haemocytometer. The protoplasts were viewed at ×100 magnification and the number of protoplasts observed was recorded. Total protoplast yield was calculated using the equation below:

$$\text{Protoplast Yield} = \frac{\text{Total cells count} \times \text{Total volume of cell suspension}}{4 \times \text{Weight of fresh tissues (g)}}$$

RESULTS AND DISCUSSION

Effects of sorbitol concentrations on protoplast isolation

An osmotic stabiliser is essential to provide osmotic support to the protoplasts following the removal of cell wall. In this study, the protoplasts obtained using the isolation solution containing 0.5 M sorbitol were more spherical in shape. In addition, the protoplasts were translucent and chloroplasts were observed in the centre of protoplasts (Figure 1A). In contrast, 1.0 M sorbitol used was found to be less effective as high osmoticum concentration could lead to the shrinking of the protoplasts (Figure 1B) because plasmolysis of protoplast might occur. According to Kanchanapoom (2001), a solution of higher concentration than the cell contents used for isolating the protoplast could cause the *D. pompadour* protoplasts to plasmolyze.

A much higher yield of protoplasts was obtained from *in vitro* leaves of *D. crumenatum* using protoplast isolation solution containing 0.5 M sorbitol compared to when 1.0 M sorbitol was used. The yield of protoplast obtained was 25.62×10^4 (±2.46) protoplasts/g FW (Figure 2) which was approximately three times higher than that of protoplast obtained by using 1.0 M sorbitol, 7.86×10^4 (±0.54) protoplasts/g FW. The osmolarity of isolation solution had a substantial effect on the yield of protoplast because

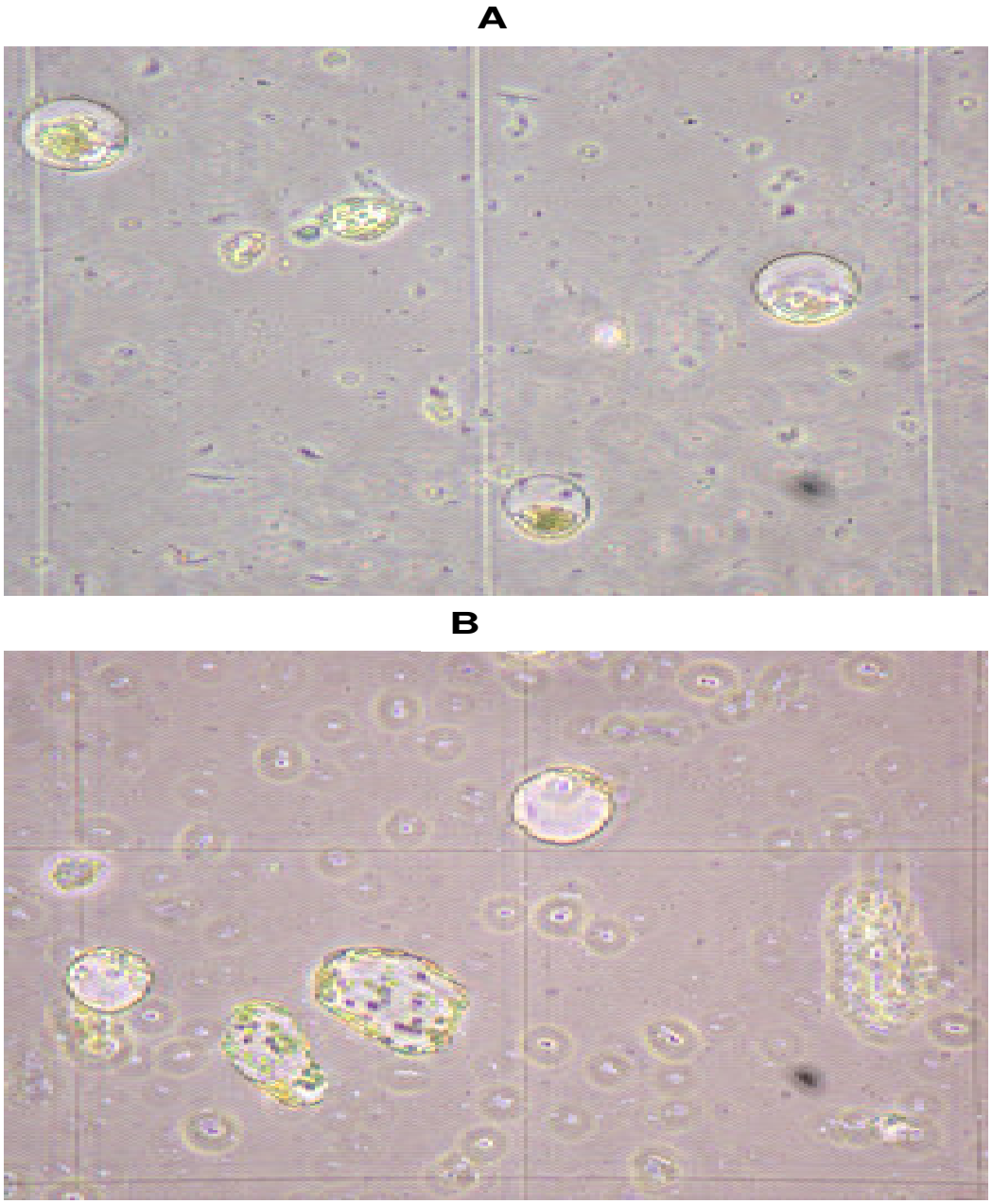

Figure 1. Effects of sorbitol concentrations on protoplast isolation in which protoplasts were isolated using protoplast isolation solution containing: (A) 0.5 M sorbitol (B) 1.0 M sorbitol. Protoplasts obtained were examined under ×200 magnification.

without sorbitol, no protoplast was isolated as shown in the control sample.

In general, the concentration of osmoticum used for protoplast isolation could be within the range of 1.0 M. For instance, 0.4 M sorbitol was the optimun concentration used to obtain high yield of protoplast for winged bean, 6.5×10^6 protoplasts/g FW of explants (Cuddihy and Bottino, 1982) and 0.6 M mannitol; and 0.6 M sorbitol was the suitable osmotic stabilizers for obtaining good protoplasts yield of Antarctic sea ice algae (Liu et al., 2006). In this study, 0.5 M sorbitol was found to be suitable for isolating the *D. crumenatum* protoplast from *in vitro* leaves.

Other than sorbitol, mannitol is also the popular omosticum used for protoplast isolation. In the study of *Dendrobium pompadour* protoplast isolation, Kanchanapoom (2001) reported that 0.4 M mannitol was suitable for protoplast release. In another study, Zhu et al. (2005) investigated the mannitol concentrations (0.6 - 0.8 M) in isolating protoplasts from *Echinacea*

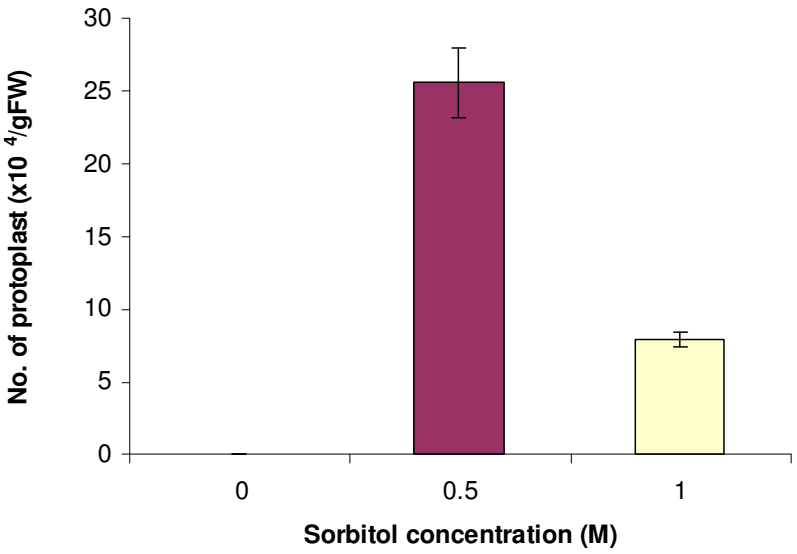

Figure 2. Effects of different sorbitol concentrations on protoplast isolation from leaves of *D. crumenatum*. Standard deviations were indicated on top of the bars.

augustifolia callus and found that 0.7 M mannitol was most suitable for the protoplast isolation, 35.0×10^4 protoplast/g FW. Besides, Loh and Rao (1985) and Koh et al. (1988), both used 0.4 M sucrose as an osmoticum in the protoplast isolation solution. The sucrose added in the wash medium was found to be effective in separating protoplasts from debris in their systems.

Effects of different combinations and concentrations of enzymes on the yield of protoplast isolation

The combination and concentration of enzymes required for complete release of protoplasts were examined. In this study, only debris of cells was observed in the control sample in which the isolation solution used was without enzyme (Figure 3A). Similarly, the isolation solution containing only pectinase was not able to isolate the protoplast as no protoplast was observed. In addition, efficacy for protoplast isolation was also very low, 2.39×10^4 (±0.57) protoplasts/g FW, when the isolation solution containing only cellulase as compared to when both pectinase and cellulase were present in the isolation solution, 20.4×10^4 (±2.17) protoplasts/g FW (Figure 4). The yield of protoplast was much higher when 2% (w/v) of both cellulase and pectinase were used, approximately twenty times higher than the yield obtained from the solution containing only cellulase. Generally, all the isolated protoplasts were spherical in shape and chloroplasts were clearly observed.

According to Prasertsongskun (2004), protoplasts were efficiently released from explants by 10 h incubation with the enzyme mixture containing 2% cellulase Onozuka R10, 2% macerozyme R10 and 0.5% pectinase, 8.4×10^4 protoplasts/ml. Prasertsongskun (2004) found that a low protoplast yield was obtained in the absence of pectinase. This showed that the presence of pectinase was essential to increase protoplast yield. Mills and Hammerschlag (1994) also reported the addition of pectinase improved the yield of viable peach protoplasts which were isolated using combinations of cellulases and Onozuka R-10 with addition of the pectinase.

The concentration of enzymes is critical for complete protoplast release. The protoplasts obtained from the isolation solution containing 2% (w/v) pectinase and cellulase were spherical in shape and well separated (Figure 5). The protoplasts were translucent and the light green chloroplasts were clearly observed inside the protoplasts. In contrast, the protoplasts were obtained from the protoplast isolation solution containing 1% (w/v) pectinase and cellulase were aggregated (Figure 5). The yield of protoplast obtained by using 2% (w/v) enzymes (pectinase and cellulase), 20.12×10^4 (±1.54) protoplasts/g FW, was more than 2-fold higher than that obtained from 1% (w/v) enzymes isolation solution, 9.06×10^4 (±1.26) protoplasts/g FW (Figure 6). This study suggested that 2% of both enzymes used were more suitable for protoplast releasing.

Our results showed the lower cellulase concentration might not able to liberate protoplasts efficiently. Similar observation was reported by Zhu et al. (2005) as higher cellulase concentration was required for protoplast isolation from *Echinacea augustifolia*. Their results showed that the yield of protoplasts was low when 1.5% (w/v) cellulase was used and 2.0% (w/v) cellulase was the optimum concentration for protoplast isolation. However,

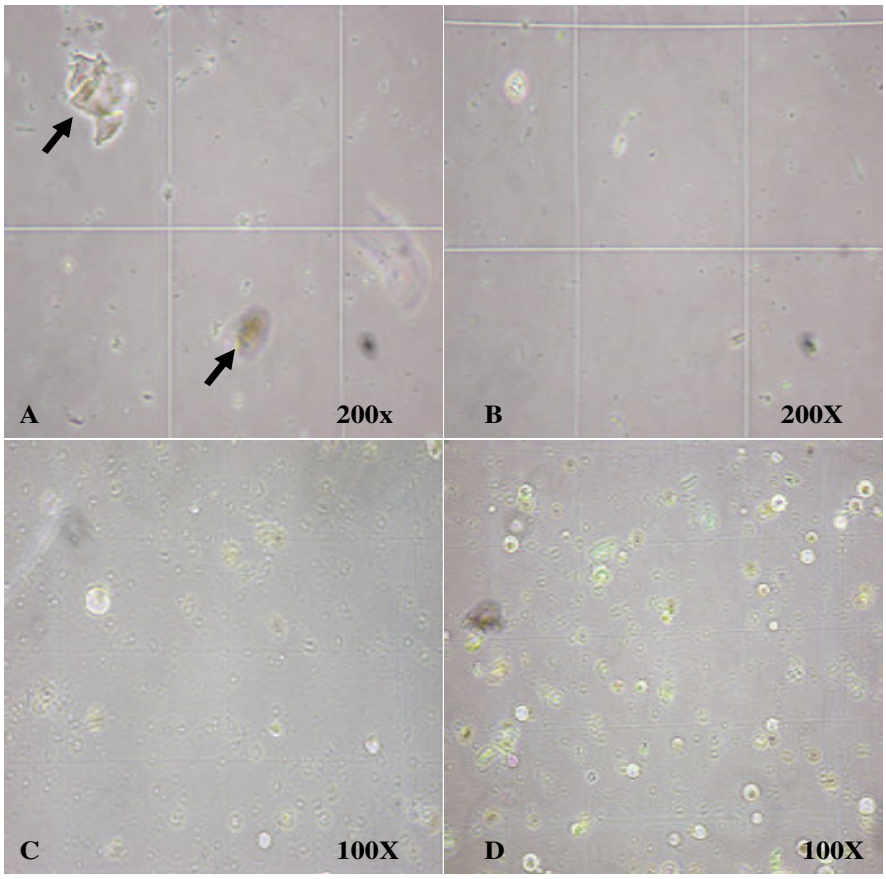

Figure 3. Effects of enzymes combinations on protoplasts isolation. (A) No protoplasts were obtained from the isolation solution without pectinase and cellullase (arrows show debris) and (B) protoplast isolation solution containing only pectinase. (C) Protoplasts were isolated using protoplast isolation solution containing cellulase only and (D) protoplast isolation solution containing cellulase and pectinase

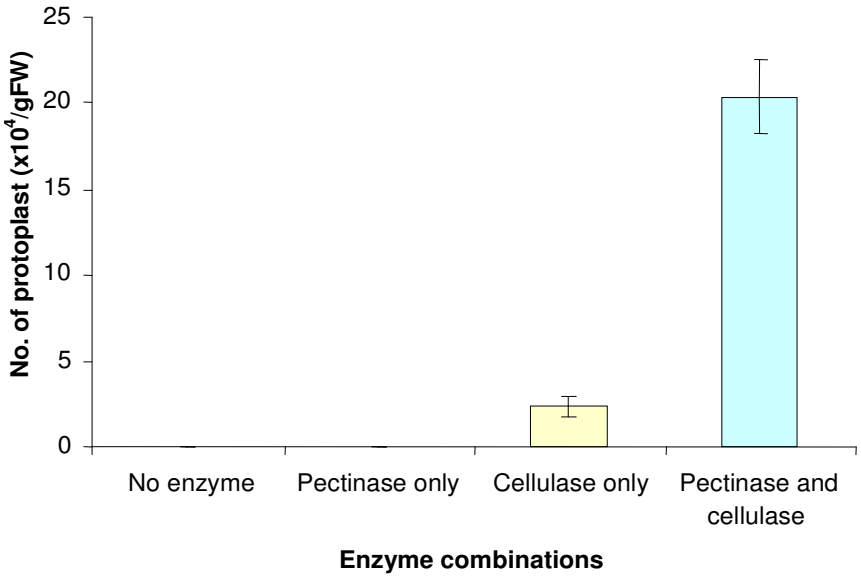

Figure 4. Effects of different enzymes combinations on protoplast isolation from leaves of *D. crumenatum.* Standard deviations were indicated on top of the bars.

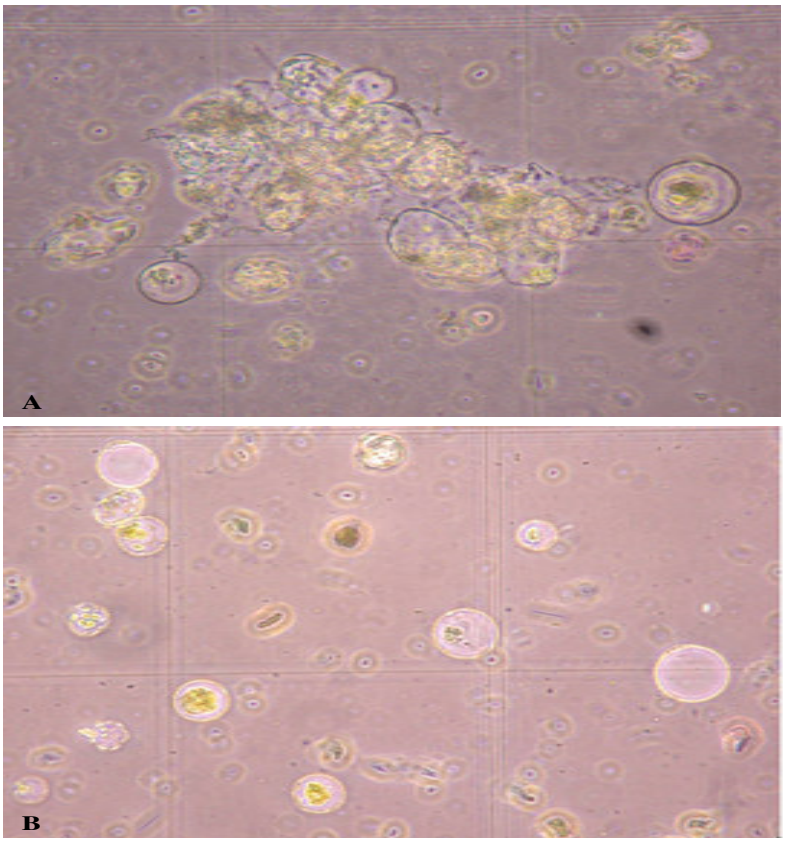

Figure 5. Effects of the concentration of enzymes on protoplasts isolation. (A) Protoplasts were isolated using protoplast isolation solution containing 1% (w/v) cellulase and 1% (w/v) pectinase. (B) Protoplasts were isolated using protoplast isolation solution containing 2% (w/v) cellullase and 2% (w/v) pectinase. The arrows show location of protoplast obtained. Protoplasts obtained were examined under ×200 magnification.

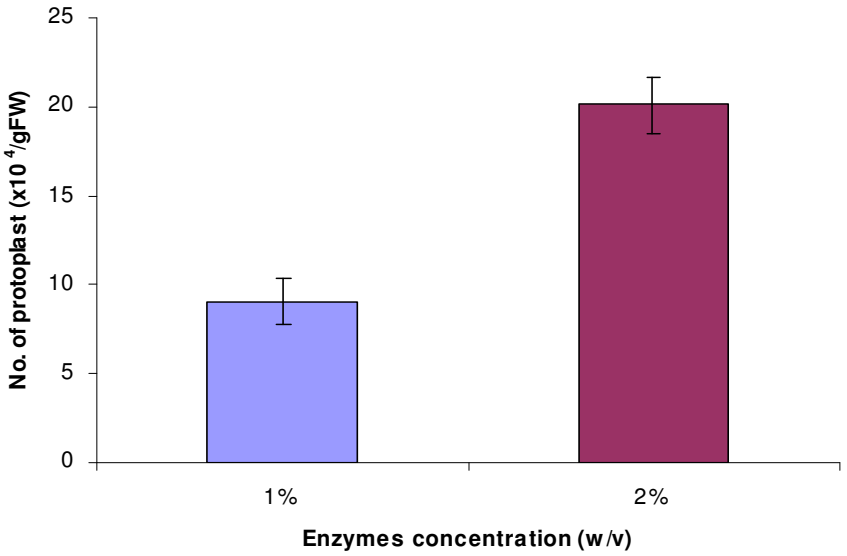

Figure 6. Effects of different concentrations of the enzymes on protoplast isolation from leaves of *D. crumenatum*. Standard deviations were indicated on top of the bars.

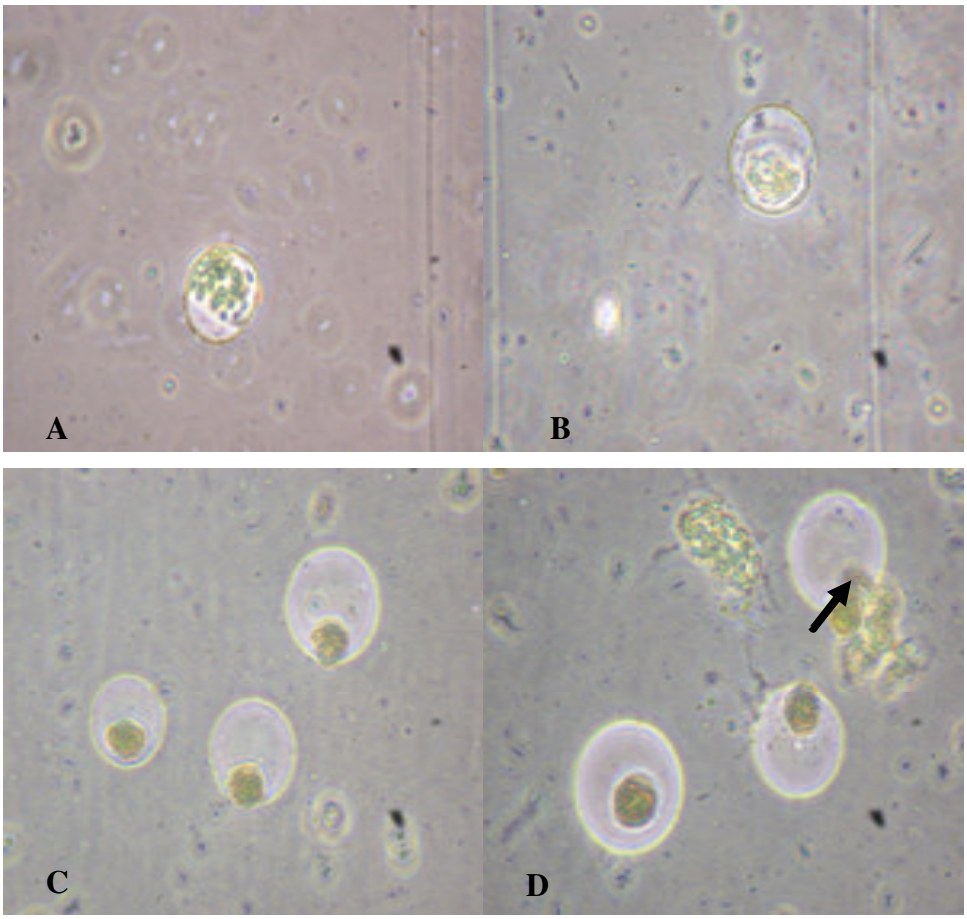

Figure 7. Effects of different incubation times with the enzymes on protoplast isolation: (A) 0 h (B) 2 h (C) 4 h (D) 6 h. The arrow shows protoplast burst after 6 h incubation. Protoplasts were examined under ×400 magnification.

Zhu et al. (2005) also reported the yield of protoplasts decreased due to the higher cellulase concentration used, 2.5% (w/v), as over-digestion of plant tissues might occur.

Effects of incubation time on the yield of protoplast isolation

In this study, the morphological appearance of protoplasts obtained was different when the incubation time was longer. The isolated protoplasts were larger and spherical in shape when the incubation times used were extended to 4 h and 6 h. In addition, the chloroplasts in these protoplasts were all aggregated in the centre (Figure 7). The protoplasts obtained after 0 h and 2 h of incubation were smaller in size and oval in shape. The chloroplasts were all scattered in the protoplasts isolated when incubated for 2 h or less than 2 h (Figure 7). The longer incubation time, 6 h, used for isolating protoplasts might cause the burst of protoplasts (Figure 7D), as chloroplasts aggregating outside of the protoplast was observed.

The yield of protoplasts increased when the incubation times used were increased t0 4 h. When the incubation time was extended to 6 h, a decrease of the protoplast yield was observed. The yield of protoplasts obtained for 0 h, 2 h and 4 h incubation time was 1.06×10^4 (±0.17) protoplasts/g FW, 11.48×10^4 (±3.00) protoplasts/g FW and 28.66×10^4 (±2.20) protoplasts/g FW, respectively (Figure 8). The 4 h incubation time employed had doubled the yield of protoplast obtained as compared to when 2 h was applied for incubation. However, the protoplast yield declined when 6 h incubation was applied, 18.84×10^4 (±3.22) protoplasts/g FW.

The incubation time, duration of incubating plant tissues in protoplast isolation solution, required for isolation of protoplasts varied among different plant species. In this study, the suitable incubation time for isolating protoplasts from the leaves of *D. crumenatum* was four hours. The highest yield of protoplasts, 28.66×10^4 protoplasts/g FW was obtained when the

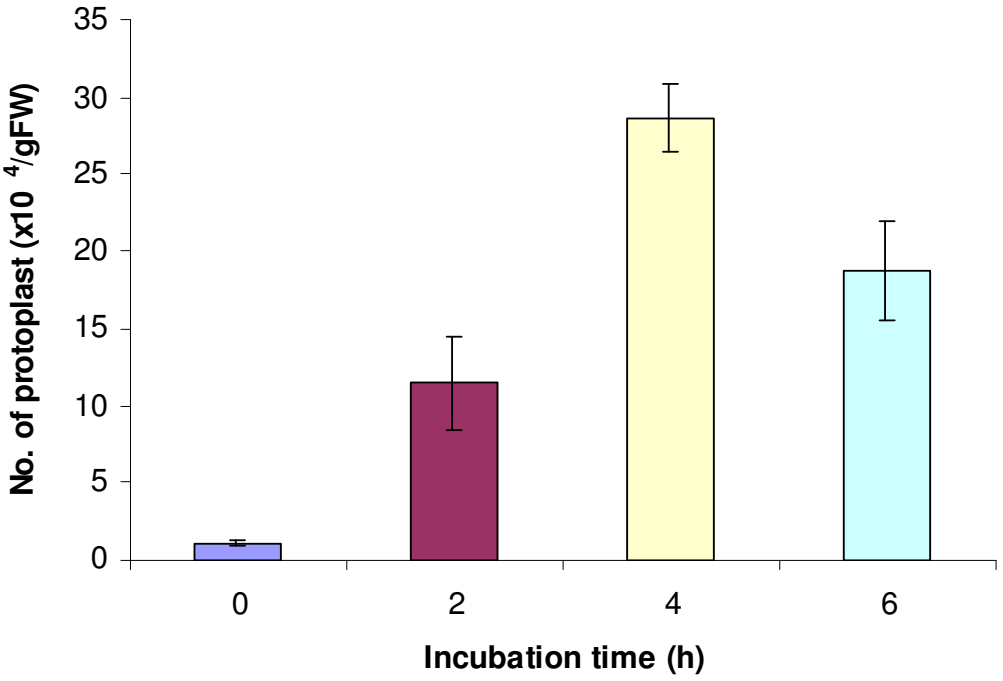

Figure 8. Effects of different incubation times on protoplast isolation from leaves of *D. crumenatum*. Standard deviations were indicated on top of the bars.

explants were incubated for 4 h compared to other incubation times examined. The yield of protoplast increased from 0 h to 4 h, but decreased when the incubation time was increased to 6 h. Zhu et al. (2005) reported that the highest yield of *E. augustifolio* protoplasts, 50.0×10^4 protoplast/g FW, was achieved when 8 h incubation time was applied as compared to other incubation times investigated, 4, 6, 10 and 12 h. The yield of protoplast declined when the incubation time was longer than 8 h. However, isolation of protoplast from *Alstroemeria* callus tissues obtained optimum yield, 19.2×10^5 protoplast/g FW, when 24 h incubation time was applied as compared to other incubation times examined, 4, 8, 12, 16, and 20 (Kim et al., 2005).

The incubation time required for releasing protoplasts could also be influenced by the enzyme concentration and compositions of the protoplast isolation solution used. For example, Balestri and Cinelli (2001) reported that protoplast isolation of *Cymodocea nodosa* from the leaves explants required 7 - 9 h incubation time using protoplast isolation solution containing 1% (w/v) cellulase and 1% (w/v) pectinase; and Nassour and Dorion (2002) also reported lower enzyme concentration required longer incubation time as the optimum incubation time for protoplast isolation from leaves of *Pelargonium* x *hortorum* 'Alain' was 6 - 12 h using protoplast isolation solution containing low enzyme concentration, 0.4% (w/v) cellulose and 0.2% (w/v) pectinase. This suggested that the longer incubation time was required when the concentration of enzymes was lower.

Conclusion

The isolation conditions are extremely important for the efficient release of protoplasts from *in vitro* leaves of *D. crumenatum*. Based on the results, 0.5 M sorbitol, the combination of 2% (w/v) cellulase and 2% (w/v) pectinase and 4 h incubation time were the most suitable conditions for protoplast isolation of *D. crumenatum* using *in vitro* leaves in the study. The established protocol could be used for future research in manipulating the genes, particularly in protoplast fusion study.

REFERENCES

Balestri E, Cinelli F (2001). Isolation and cell wall regeneration of protoplasts from *Posidonia oceanica* and *Cymodocea nodosa*. Aquatic Botany, 70: 237-242.

Beaman TE, Wood JJ, Beaman RS, Beaman JH (2001). *Orchids of Sarawak*. Malaysia: Natural History Publication (Borneo) Sdn. Bhd, Malaysia, p. 32: 242

Cuddihy AE, Bottino PJ (1982). Winged-bean protoplasts: isolation and culture to callus. Plant Cell, Tissue and Organ Cult., I: 201-209.

Kanchanapoom K, Jantaro S, Rakchad D (2001). Isolation and fusion of protoplasts from mesophyll cells of *Dendrobium Pompadour*. Science Asia, 27: 29-34.

Kim JB, Bergervoet JEM, Raemakers JJM, Jacobsen E, Visser RGF (2005). Isolation of protoplasts, and culture and regeneration into plants in *Alstroemeri*a. *In Vitro* Cellular and Developmental Biol., 41: 505-510.

Koh MC, Goh CJ, Loh CS (1988). Protoplast isolation and culture of *Aranda* hybrids. Malayan Orchid Rev., 22: 70-8.

Liu SH, Liu CL, Huang XH, Chai YM, Cong BL (2006). Optimization of parameters for isolation of protoplasts from the Antarctic sea ice alga

Chlamydomonas sp. ICE-L. J. Appl. Phycol., 18: 783-786.
Loh CS, Rao AN (1985). Isolation and culture of mesophyll protoplast of *Aranda* Noorah Alsagoff. Malayan Orchid Rev., 19: 34-7.
Mills D, Hammerschlag FA (1994). Isolation of cells and protoplasts from leaves of *in vitro* propagated peach *(Prunus persica)* plants. Plant Cell, Tissue and Organ Cult., 36: 99-105.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissu culture. Physiol. Plant, 15: 473- 497
Nassour M, Dorion M (2002). Plant regeneration from protoplasts of micropropagated *Pelargonium x hortorum* 'Alain': Effect of some environmental and medium factors on protoplast system efficiency. Plant Sci., 163: 169-173.
Oshiro MA, Steinhart WL (1991). Preparation of protoplasts from cells of orchids representing various genera. Lindleyana, 6(1): 36-41.
Papadakis AK, Roubelakis-Angelaskis KA (2002). Oxidative stress could be responsible for the recalcitrance of plant protoplasts. Plant Physiol. Biochem., 40: 549-559.
Prasertsongskun S (2004). Isolation and culture of suspension protoplasts of vetiver. J. Sci. Technol., 26(3): 411-416.
Price GR, Earle ED (1984). Sources of orchid protoplasts for fusion experiments. Am Orchid Soc Bull., 53: 1035-43.
Sajise JU, Sagawa Y. (1991). Regeneration of plantlets from callus and protoplasts of *Phalaenopsis* sp. Malayan Orchid Bull., 5: 23-8.
Teo CKH, Neumann KH (1978). The culture of protoplasts isolated from *Renantanda* Rosalind Cheok. Orchid Rev., 86:156-58.
Zhu L, Wang BC, Zhou J, Chen LX, Dai CY, Duan CR (2005). Protoplast isolation of callus in *Echinacea augustifolia*. Colloids and Surfaces B: Biointerfaces, 44: 1-5.