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(RESEARCH ARTICLE)

Improvements in Protoplast Isolation Protocol of *Dendrobium antennatum* Lindl

Reni Mahmudah, Edy Setiti Wida Utami, Yosephine Sri Wulan Manuhara, Junairiah and Sucipto Hariyanto *

Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Surabaya 60115, Indonesia.

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Abstract

This study aimed to determine the effect of a combination of enzyme concentrations, incubation time, and osmoticum concentrations on protoplast isolation mesophyll of leaves of *Dendrobium antennatum* Lindl. This was an experimental study using a 3×3×3 factorial design and three replications.The treatment variable was a combination of 3 levels of enzyme concentration (0.5% *cellulase* + 0.2% *pectinase*, 1% *cellulase* + 0.2% *pectinase*, and 1.5% *cellulase* + 0.2% *pectinase*), 3 levels of long incubation time (6 hours, 9 hours, and 12 hours), and 3 levels of osmoticum concentration (0.3 M sucrose, 0.4 M sucrose, and 0.5 M sucrose). The data on protoplast density and the number of viable protoplasts were analyzed using the Kruskal-Wallis test followed by the Whitney test. The results showed that the combination of the three treatment variables influences protoplast density and the number of viable protoplasts. The highest protoplast density was achieved in the combined treatment (1.5% *cellulase* + 0.2% *pectinase*), 6 hours of incubation time, and 0.3 M sucrose. The highest number of viable protoplasts was achieved in the combined treatment (0.5% *cellulase* + 0.2% *pectinase*), 6 hours of incubation time, and 0.5 M sucrose.

Keywords: Protoplasts isolation; *Dendrobium antennatum* Lindl; Cellulase; Pectinase

1. Introduction

Commercial orchids currently under development are generally hybrid plants derived from crossing products from foreign countries, such as Thailand, Taiwan, Singapore, Hawaii, and Australia. Indonesia is far behind in the field of breeding and the orchid industry, even though there are quite a lot of genetic resources available. Orchid businesses remain predominantly dominated by small farmers. However, its development is hindered by several challenges, including the limited supply of superior seeds selected from commercial cultivars, the relatively expensive price of superior seeds, and a lack of market information and capital. Orchids are generally used as ornamental plants and as cut flowers, these include [1]. *Dendrobium* is a genus in the Orchidaceae family. The flowers are particularly notable for their high commercial value and wide geographical distribution [2]. Several *Dendrobium* hybrids are important to the cut flower industry in a number of countries and are also popular among orchid growers [3]. *Dendrobium* hybrids have a variety of flower colors and color patterns. In addition, the relatively short production cycle from seed to flowering plant can provide valuable and highly desirable characteristics for large-scale commercial production [4]. *Dendrobium* orchids are easy to grow, capable of flowering continuously, and available in a wide range of colors. Their flexible stems make them simple to arrange, and the flowers are long-lasting. These traits, combined with short production times, enable *Dendrobium* orchids to adapt to the changing preferences of flower consumers. Many *Dendrobium* cultivars and hybrids originate from conventional crosses and somaclonal variations resulting from tissue culture techniques. Although there are many interspecific *Dendrobium* hybrids, it is difficult to produce intergeneric hybrids using conventional hybridization techniques. Somatic hybridization through protoplast fusion allows combination and hybridization in different genera and species that are sexually incompatible [5]. Conventional orchid hybridization techniques cannot easily manipulate specific flower traits and other desirable characteristics such as life stage and flowering time to compete with consumer demand [6]. To achieve success in the implementation of the use of protoplasts as a target for somatic hybridization and direct gene transfer for the improvement of new *Dendrobium*

* Corresponding author: Sucipto Hariyanto

hybrids, orchid protoplasts obtained through protoplast isolation must be viable and culturable [7]. Improvements in plant quality obtained from protoplast manipulation can be used in breeding programs to develop new cultivars [8]. In addition, the development of protoplast systems has increased the flexibility of plants for use in both biochemical and genetic studies. Protoplasts are indispensable in genetic engineering and plant breeding [9]. Protoplast culture has been used to develop plants with improved agronomic and horticultural characteristics and increased disease resistance through somatic hybridization and plant genetic engineering. By isolating and regenerating plants from protoplasts, variants (soma-clones) with improved characteristics can be obtained. Parasexual and somatic hybrids can be obtained by fusing protoplasts of unrelated or distantly related species. Through genetic engineering, foreign genes can be transferred to plant protoplasts and enhance regeneration with new genes in plants from transformed cells [8]. For plant regeneration using protoplasts, an efficient protocol for isolating plant protoplasts is essential [10]. Isolated protoplasts are described as “naked” plant cells because the cell wall has been experimentally removed through an enzymatic process [11]. The pectinase enzyme is used to separate tissue into single cells, and the cellulase or hemicellulase enzyme is used to lyse cell walls, which produces viable protoplasts [12]. When protoplast isolation is carried out using the enzymatic method, it is necessary to add a suitable osmotic solution to the enzyme solution. Osmotic solutions are needed to stabilize and prevent protoplast membranes from breaking [13]. To date, there are few reports on the isolation and culture of protoplasts from *Dendrobium* [7, 10]. According to Tee [14], in the practice of protoplast isolation, each species requires a different enzyme concentration, incubation time, and osmotic concentration. Therefore, this study emphasizes optimization in the isolation of *Dendrobium antennatum* Lindl protoplasts to provide basic data in efforts to improve and increase the quality of *Dendrobium*.

2. Material and methods

2.1. Explant source

This study was carried out at the Plant Physiology Laboratory, Department of Biology, Faculty of Science and Technology, and at the Institute for Tropical Diseases, Universitas Airlangga.

D. lineale plantlets were obtained from "DD Orchid Nursery" Batu, East Java, Indonesia. The explant source is the second and third order leaves of the shoot, and the leaf mesophyll is used as explant material.

2.2. Protoplast isolation

In this study, 1 gram of leaf was taken from *Dendrobium antennatum* Lindl plantlets. The upper and lower epidermis layers of the leaves were incised. The explants are then cut transversely ± 1 mm with a blade on a Petri dish and then put into a vial containing an enzyme solution (E1 = 0.5%cellulose + 0.2% pectinase, E2 = 1%cellulase + 0.2% pectinase, E3 = 1.5%cellulase + 0.2% pectinase) and sucrose as an osmoticum (0.3 M, 0.4 M, and 0.5 M).

The vial tube was covered with aluminum foil and incubated in the dark for 6 hours, 9 hours, and 12 hours respectively, then filtered through a nylon filter to separate uncut tissue and debris. Protoplasts are washed in a washing solution by picking up protoplasts floating on the surface using a micropipette.

Protoplasts were then purified by transferring the floating protoplasts using a micropipette from the washing solution into the purifying solution [7, 15]. The density and number of viable protoplasts were obtained by counting using a hemacytometer under a light microscope. Protoplasts were viewed at ×100 magnification, and the number of protoplasts observed was recorded [14]. The number of protoplasts observed was calculated using the following equation [16]:

$$S = \frac{\overline{x}}{L \times t \times P} \times 10^3$$

S = number of protoplasts
$\overline{x}$ = the average number of protoplasts from the chambers observed
L = 1 mm^2 (haemocytometer area 1 mm^2/25 counting chambers×25 counting chambers)
t = 0.1 mm (hemocytometer height)
P = dilution
10^3 = conversion constant from mm^3 to Ml

Viability testing was determined under a fluorescent microscope. Protoplasts showing green/yellow fluorescence were declared viable [17]. Protoplasts used for viability testing were derived from the treatment that demonstrated the most significant impact on the number of viable protoplasts.

2.3. Statistical analysis

The experimental units were set up in a completely randomized factorial design. The data were analyzed with SPSS (Version 23) using analysis of variance (ANOVA). The mean values were separated using Mann Whitney with the level of significance at ≤ 0.05.

3. Results

In this study, explants were taken from the mesophyll of the leaves of *Dendrobium antennatum* Lindl orchid growing in in vitro conditions (Figure 1). Protoplasts isolated enzymatically are present on the surface of the washing solution (Figure 2). Protoplasts floating on the surface are then observed to determine protoplast density and the number of viable protoplasts.

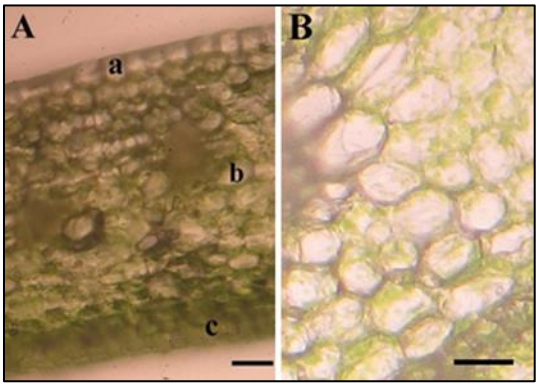

Figure 1 Cross section of *Dendrobium antennatum* Lindl leaf. (A) Leaf tissue: a. upper epidermis, b. mesophyll, c. lower epidermis; (B) Mesophyll tissue before isolation: mesophyll cells are hexagon-shaped. Bar = 50 µm

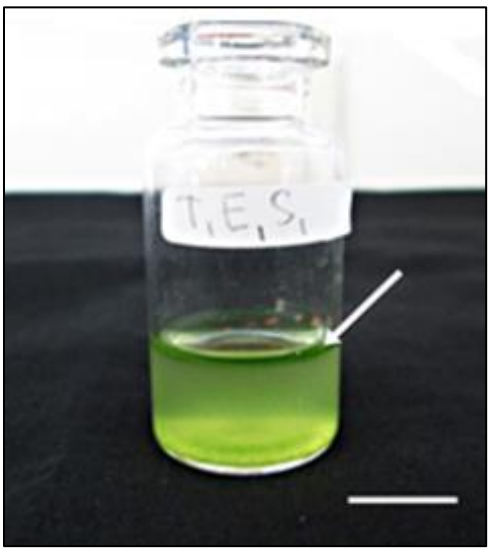

Figure 2 Protoplasts in washing solution containing sucrose osmoticum. The arrow shows protoplast solution floating on the surface. Bar = 1 cm

Protoplast density is the total number of viable protoplasts and non-viable protoplasts per mL of solution presented in Table 1, and the average viable protoplasts are presented in Table 2. Viable protoplasts have a perfectly round shape (Figure 3A), while non-viable protoplasts have a shaped that is not round (Figures 3B and 3C).

Table 1 Average protoplast density isolated from the mesophyll of *Dendrobium antennatum* Lindl orchid leaves after incubation in a combination of enzyme concentration, incubation time, and osmotic concentration (number of protoplasts × 10^2 protoplasts/mL ± SD)

Treatment (n=3)		T_1	T_2	T_3
S_1	E_1	$13.3^{abcd} \pm 1.8$	$8.0^{bcde} \pm 3.2$	$5.3^{de} \pm 1.8$
	E_2	$12.0^{bcde} \pm 3.2$	$6.6^{cde} \pm 1.8$	$4.0^{e} \pm 0.0$
	E_3	$21.3^{a} \pm 4.9$	$14.6^{abc} \pm 4.9$	$12.0^{bcde} \pm 3.2$
S_2.	E_1	$8.0^{bcd} \pm 0.0$	$5.3^{de} \pm 1.8$	$5.3^{de} \pm 1.8$
	E_2	$16.0^{ab} \pm 3.2$	$12.0^{bcde} \pm 3.2$	$4.0^{e} \pm 0.0$
	E_3	$6.6^{cde} \pm 1.8$	$4.0^{e} \pm 0.0$	$4.0^{e} \pm 0.0$
S_3	E_1	$12.0^{bcde} \pm 0.0$	$9.3^{bcde} \pm 1.8$	$5.3^{de} \pm 1.8$
	E_2	$9.3^{bcde} \pm 1.8$	$5.3^{de} \pm 1.8$	$5.3^{de} \pm 1.8$
	E_3	$5.3^{de} \pm 1.8$	$4.0^{e} \pm 0.0$	$4.0^{e} \pm 0.0$

T1 = incubation time 6 hours, T2 = incubation time 9 hours, T3 = incubation time 12 hours, S1 = sucrose 0.3 M, S2 = sucrose 0.4 M, S3 = sucrose 0.5 M, E1 = 0.5% cellulase + 0.2% pectinase, E2 = 1% cellulase + 0.2% pectinase, E3 = 1.5% cellulase + 0.2% pectinase.

Mean numbers followed by the same letter indicate there is no significant difference according to the Mann-Whitney test ($p<0.05$).

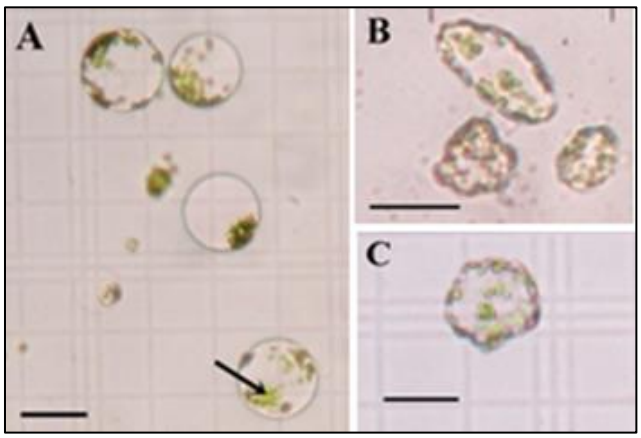

Figure 3 Isolated protoplast. (A) a viable protoplast; the arrow indicates the chloroplast; (B) and (C) non-viable protoplasts. Bar = 50 µm

Table 1 shows that there are differences in protoplast density from various treatment combinations of enzyme concentration, incubation time, and osmotic concentration used. The combination of a 1.5% cellulase + 0.2% pectinase enzyme solution, a 6-hour incubation time and a 0.3 M sucrose osmoticum produced a maximum protoplast density of 21.3 × 10^2 protoplasts/mL.

Table 2 Average number of viable protoplasts isolated from the mesophyll of *Dendrobium antennatum* Lindl orchid leaves after incubation in a combination of enzyme concentration, incubation time, and osmotic concentration (number of viable protoplasts × 10^2 protoplasts/mL ± SD)

Treatment (n=3)		T_1	T_2	T_3
S_1	E_1	$8.0^{ab} \pm 0.0$	$5.3^{abcd} \pm 2.3$	$4.0^{bcd} \pm 0.0$
	E_2	$6.6^{abc} \pm 2.3$	$4.0^{bcd} \pm 0.0$	$2.6^{bcd} \pm 2.3$
	E_3	$8.0^{ab} \pm 4.0$	$6.6^{abc} \pm 2.3$	$2.6^{bcd} \pm 2.3$
S_2	E_1	$4.0^{bcd} \pm 0.0$	$1.3^{cd} \pm 2.3$	$4.0^{bcd} \pm 0.0$

	E_2	$6.6^{abc} \pm 2.3$	$6.6^{abc} \pm 2.3$	$2.6^{bcd} \pm 2.3$
	E_3	$4.0^{bcd} \pm 0.0$	$2.6^{bc} \pm 2.3$	$0.0^{d} \pm 0.0$
S_3	E_1	$10.6^{a} \pm 2.3$	$6.6^{abc} \pm 2.3$	$5.3^{abcd} \pm 2.3$
	E_2	$4.0^{bcd} \pm 0.0$	$4.0^{bcd} \pm 0.0$	$2.6^{bcd} \pm 2.3$
	E_3	$1.3^{cd} \pm 2.3$	$0.0^{d} \pm 0.0$	$0.0^{d} \pm 0.0$

T1 = incubation time 6 hours, T2 = incubation time 9 hours, T3 = incubation time 12 hours, S1 = sucrose 0.3 M, S2 = sucrose 0.4 M, S3 = sucrose 0.5 M, E1 = 0.5% Cellulase + 0.2% Pectinase, E2 = 1% Cellulase + 0.2% Pectinase, E3 = 1.5% Cellulase + 0.2% Pectinase.

Mean numbers followed by the same letter indicate there is no significant difference according to the Mann-Whitney test ($p<0.05$).

Table 2 shows that there are differences in the number of viable protoplasts from various combinations of enzyme treatment, incubation time, and osmotic concentration used. The combination of 0.5% cellulase + 0.2% pectinase enzyme solution, a 6-hour incubation time, and a 0.5 M sucrose osmoticum produced the maximum number of viable protoplasts, namely 10.6×10^2 protoplasts/mL. In this treatment (T1S3E1), protoplasts were found to undergo cellular division (Figure 4A) and spontaneous membrane fusion (Figure 4B).

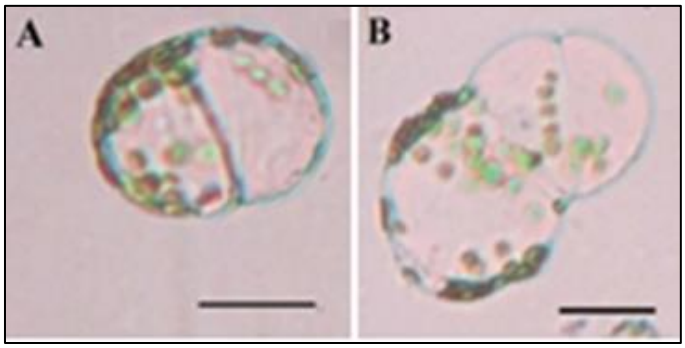

Figure 4 Protoplast undergoing cellular division and spontaneous membrane fusion. (A) Protoplast undergoing cellular division. Bar = 30 µm; (B) Protoplast undergoing spontaneous membrane fusion. Bar = 26.67µm

The protoplast viability test serves as a subsequent evaluation following morphological observation of protoplasts. Viability testing aims to strengthen the assumption that viable protoplasts are round, while non-viable protoplasts are not round. The results of obtaining the highest viable protoplasts (T1S3E1), were used in protoplast viability testing (Figure 5).

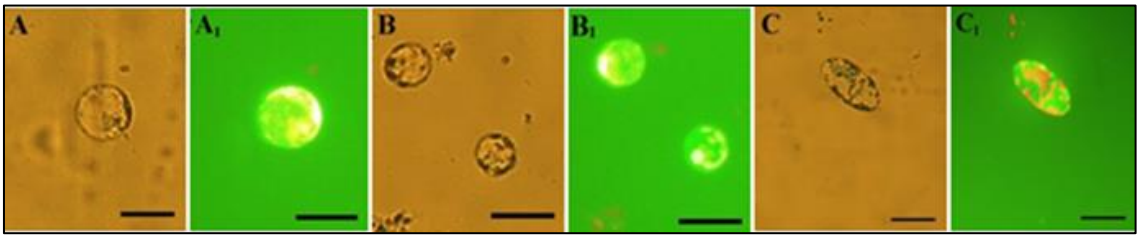

Figure 5 Protoplast viability test. (A, B) Protoplasts before staining with FDA appear round indicating viable protoplasts; (A1, B1) Viable protoplasts after staining with FDA. Protoplast appears round and fluoresces greenish yellow; (C) Protoplasts before staining with FDA do not appear round, indicating non-viable protoplasts; (C1) Non-viable protoplast after staining with FDA. Protoplast is not round and has a reddish color, indicating that protoplast is not viable. Bar = 50 µm

4. Discussion

In this study, protoplast density was obtained from the sum of the number of viable protoplasts and the number of non-viable protoplasts. According to Table 1, the isolation results from the mesophyll of orchid leaves *Dendrobium antennatum* Lindl produced a maximum protoplast density of 21.3×10^2 protoplasts/mL, obtained in the combined enzyme of 1.5% cellulase + 0.2% pectinase, 0.3 M sucrose, and an incubation time of 6 hours. Tee [14] also isolated

protoplasts from *Dendrobium crumenatum* leaves. The highest protoplast density was 28.6×10^4 protoplasts/mL, obtained with a combination of 2% cellulase + 2% pectinase, 0.5 M sorbitol osmotic, and a 4-hour incubation time.

In this study, low cellulase and pectinase concentrations with long incubation times were unable to release more protoplasts. However, high cellulase concentrations had an impact on membrane integrity and reduced physiological activity and could even cause over-digestion of plant material [18]. This is confirmed by Duquenne's statement [19], which stated that increasing cellulase concentrations negatively affected the viability of protoplasts isolated from *Spathiphyllum wallisii* 'Alain' and *Anthurium scherzerianum* embryos.

The use of 0.3 M sucrose (S1) resulted in the highest protoplast density. In the T1S1E3 treatment, the number of viable protoplasts was lower at 8.0×10^2 protoplasts/mL, while the number of non-viable protoplasts was higher at 13.3×10^2 protoplasts/mL. Protoplasts in this treatment failed to undergo cell division and did not exhibit spontaneous membrane fusion. A similar outcome was observed in the treatment using 0.4 M sucrose (S2) as the osmotic agent. The use of sucrose in the washing medium is known to be effective in separating protoplasts from debris during protoplast isolation. In addition, sucrose can reduce rapida crystals released from idioblasts during cell wall destruction. *Dendrobium mesophyll* cells are similar to other plant families, including Rubiaceae, Acanthaceae, and Malvaceae, which contain idioblast cells. Eliminating the damaging effects of these crystals is important because the sharpness of the needle-like structures can puncture and break protoplasts [10].

In the incubation time, the highest total protoplast density (T1S1E3) was 6 hours but produced a lower number of viable protoplasts of 8.0×10^2 protoplasts/mL and a higher number of non-viable protoplasts of 13.3×10^2 protoplasts/mL. With incubation times of 9 and 12 hours, the overall protoplast density decreased, both in terms of the number of viable protoplasts and the number of non-viable protoplasts. Prolonged incubation times have the potential to cause cell damage. This is different from the investigation by Grzebelus [20] on carrot plants with an incubation time of 14-16 hours producing a high number of viable protoplasts. For short incubation times, the concentration of the enzyme solution can be increased, and for longer incubation times, the concentration of the enzyme solution can be reduced [21, 22].

According to Table 2, the results of protoplast isolation in the mesophyll of *Dendrobium antennatum* Lindl orchid leaves produced a maximum number of viable protoplasts of 10.6×10^2 protoplasts/mL, obtained from the enzyme of 0.5% cellulase + 0.2% pectinase, 0.5 M sucrose, and an incubation time of 6 hours. When the cellulase concentration is low, the number of protoplasts produced shows a better response, whereas, at high enzyme concentrations, it causes damage to plant protoplasts. This is in line with Khentry's investigation [7]: an enzyme concentration of 1.5% cellulase + 0.2% pectinase with an incubation time of 4 hours obtained a protoplast number of 5.3×10^5 protoplasts/mL with lower protoplast viability of 92.6% compared to an enzyme concentration of 1% cellulase + 0.2% pectinase, which obtained a protoplast number of 3.9×10^5 protoplasts/mL, which had a viability of protoplasts that was higher (95.6%).

In the sucrose concentration treatment, the use of 0.5 M sucrose (S3) produced the highest viable protoplasts. Some protoplasts at this concentration experienced membrane fusion and cellular division (Figure 4). The osmotic gradient in this treatment shows the direction of membrane fusion as in Kanchanapoom's investigation [10]. During isolation, spontaneous membrane fusion can occur between two or more adjacent protoplasts. The process appears to occur when the plasmodesmata between 2 protoplasts fuse together. When protoplasts fuse, the cell nucleus and cytoplasmic material from the 2 protoplasts will fuse into one. Spontaneous fusion of different cell sources and species is rare [11, 13].

In the incubation time treatment, the highest number of viable protoplasts (T1S3E1) was 6 hours, producing several viable protoplasts of 10.6×10^2 protoplasts/mL. With incubation times of 9 and 12 hours, the overall number of viable protoplasts decreased. This is in line with Raikar's investigation [23], in which the isolation of *Lotus corniculatus* protoplasts with an incubation time of 6 hours yielded 7.2×10^6 protoplasts/mL with high protoplast viability, namely 76%, whereas in the treatment with an incubation time of 8 hours, 7.4×10^6 protoplasts/mL were obtained with protoplast viability being 52% lower.

In the protoplast viability test (Figure 5), viable protoplasts after staining with FDA appeared round and fluoresced greenish yellow, while non-viable protoplasts after staining with FDA were not round and had a reddish color. In viable protoplasts, the fluorescein ester molecules in FDA accumulate in the cell and are not removed from the cell membrane because the cell membrane is still intact. Meanwhile, in non-viable protoplasts, the fluorescein ester molecule in FDA is removed from the cell because the cell membrane is no longer intact or has been damaged, thereby viable cells in this staining appear to show greenish-yellow fluorescence, while non-viable cells are reddish in color.

5. Conclusion

In this study, we investigated the effects of enzyme concentration, incubation time, and sucrose concentration on the protoplasm isolation of *Dendrobium antennatum* Lindl. We found that the combination of enzyme treatment of 0.5% cellulase + 0.2% pectinase, 0.5 M sucrose, and an incubation time of 6 hours is the optimal combination for isolating protoplasts from the mesophyll of *Dendrobium antennatum* Lindl orchid leaves.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest.

References

[1] Arditti, J. (1992). Fundamentals of Orchid Biology, John Wiley and Sons Inc., New York. 691.

[2] Meesawat, U., Srisawat, T., Eksomtramage, L., & Kanchanapoom, K. (2008). Nuclear DNA content of the pigeon orchid (Dendrobium crumenatum Sw.) with the analysis of flow cytometry. Songklanakarin J. Sci. Technol, 30(3), 277-280.

[3] Arditti, J., & Ernst, R. (1993). Micropropagation of Orchids, John Wiley and Sons Inc., New York. 682.

[4] Vendrame, W.A., Carvalho, V.S., Dias, J.M.M., & Maguire, I. (2008). Pollination of Dendrobium hybrids using cryopreserved pollen. Hort Science, 43(1), 264-267.

[5] Davey, M.R., Anthony, P., Power, J.B., & Lowe, K.C. (2005). Plant protoplasts: status and biotechnological perspectives. Biotech. Adv, 23, 131-171.

[6] Chai, D., & Yu, H. (2007). Recent advances in transgenic orchid production. Orchid Science and Biotechnology, 1(2), 34-39.

[7] Khentry, Y., Paradornuvat, A., Tantiwiwati, S., Phansiri, S., & Thaveechai, N. (2006). Protoplast isolation and culture of Dendrobium sonia "Bom 17". Journal Nature Science, 40, 361-369.

[8] Trigiano, R.N., & Gray, D.J. (2000). Plant Tissue Culture Concepts and Laboratory Exercises, 2nd edition., CRC Press., Boca Raton, USA, 454.

[9] Rao, K.S. & Prakash, A.H. (1995). A simple method for the isolation of plant protoplasts. J.Biosci, 20, 645-655.

[10] Kanchanapoom, K., Jantaro, S., & Rakchad, D. (2001). Isolation and fusion of protoplasts from mesophyll cells of Dendrobium pompadour. Sci. Asia, 27, 29-34.

[11] Dodds, J.H & Roberts, L.W. (1995). Experiments in Plant Tissue Culture, 3rd edition., Cambridge University press., Cambridge, 276.

[12] Smith, R. H. (2000). Plant Tissue Culture Techniques and Experiments, 2nd edition., Academic press., A Harcourt Science and Technology Company, USA, 231.

[13] Torres, K.C. (1989). Tissue Culture Techniques for Holticultural Crops, Van Nostrand Reinhold, New York. 285.

[14] Tee, C.S., Lee, P.S., Kiong, A.L.P., & Mahmood, M. (2010). Optimisation of protoplasts isolation protocols using in vitro leaves of Dendrobium crumenatum (pigeon orchid), African Journal of Agricultural Research. 5(19), 2685-2693.

[15] Machmudi M ., Purnobasuki, H., & Utami, E.S.W. (2019). The optimization mesophyll protoplast isolation for Phalaenopsis amboinensis. Bulgarian Journal of Agricultural Science, 25 (4), 737–743.

[16] Marienfield laboratory glassware. (2010). Marienfield Info Counting Chamber, http://www.marienfeld-superior.com/index.php/manuals.html.

[17] Babaoglu, M. (2000). Protoplast isolation in Lupin (Lupinus mutabilis Sweet): determination of optimum explant sources and isolation conditions. Turk J Bot, 24, 177-185.

[18] Mukhtar, I., Bajwa, R., & Nasim, G. (2012). Isolation of mesophyll protoplasts from leaves of Dalbergia sisso Roxb. J. Appl. Sci. Environ, 16(1), 11-15.

[19] Duquenne, B., Eeckhaut, T., Werbrouck, S., & Huylenbroeck, J.V. (2007). Effect of enzyme concentrations on protoplasts isolation and protoplast culture of Spathiphyllum and Anthurium. Plant Cell Tiss Organ Cult, 91, 165-173.

[20] Grzebelus E., Szklarcyk, M., & Baranski, R. (2011). An improved protocol for plant regeneration from leaf- and hypocotyl-derived protoplasts of carrot. Plant Cell Tiss Organ Cult, DOI 10.1007/s11240-011-0078-5.

[21] Pongchawee, K., Nakorn, U.N., Lamseejan, S., Poompuang, S., & Phansiri, S. (2006). Factors affecting the protoplast isolation and culture of Anubias nana Engler. International Journal of Botany, 2(2), 193-200.

[22] Lord, C.E.N., & Gunawardena, A.H.L.A.N. (2010). Isolation of leaf protoplast from the submerged aquatic monocot Aponogeton madagascariensis. The American Journal of Plant Science and Biotechnology, 4(2), 6-11.

[23] Raikar, S.V., Braun, R.H., Bryant, C., Conner, A.J., & Christey, M.C. (2008). Efficient isolation, culture and regeneration of Lotus corniculatus protoplasts. Plant Biotechnol Rep, 2, 171-177.