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A STUDY ON THE ISOLATION OF PROTOPLASTS FROM MESOPHYLL CELLS OF *DENDROBIUM* QUEEN PINK

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Abstract

Protoplasts were successfully isolated from one month old *In vitro* grown plantlets of *Dendrobium* cultivar *Queen pink*. The enzyme solution used was composed of 1% Cellulase Onozuka R-10, 0.5% Macerozyme R-10, 0.1% Pectinase, 0.3 M mannitol, 10 mM $CaCl_2.2H_2O$ and 10 mM 2 (*N*-morpholino)-ethanesulfonic acid (MES) at pH 5.8. Protoplast highest yield with $15.7x10^4$ protoplasts per 1.5 gm freshly chopped leaves were obtained when digested in enzyme solution for 4 hrs on a rotary shaker with an agitation speed of 45 rpm in dark conditions. Protoplasts were filtered with 45µm nylon sieve and 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 gradient. Purification of protoplasts on a sucrose mannitol gradient yielded clean protoplasts that were free from debris.

Key words: Protoplast isolation protocol, *Dendrobium* Queen Pink, Protoplast yield, Protoplast viability**.**

Introduction

Orchidaceae family has more than 1000 genera and approximately 22,000 species (Dodson, 2016). Orchids occur throughout the world, they are abundant in tropical regions of America, Africa and Asia, as these are the main regions where orchids are cultivated on large scale. Orchids are most remarkable due to their diversity and floral structure. Most of orchid varieties are epiphytic, they use rocks or static objects for support and derive their nutrients and water from the atmosphere and debris, and however some species grow in the ground, under forest or grassland areas (Robert & Calaway, 1960).

Orchidaceae has gained some special significance among other cut flower families in recent times from horticultural point of view. *Dendrobium* cultivars are available in all seasons throughout the year especially autumn because it takes relatively lesser time for its growth. *Dendrobium*s has great economic value and are commercially profitable flowers in the world which is suitable for making bouquets and flower arrangements because of their vibrant colors like yellow, purple, white and pink due to its long vase life (Yuphin *et al*., 2006). Orchids are not only ornamental plants but also serve as a source of traditional medicines. Boiled bulbs of *Bletia purpurea* and its liquid helps to treat the poisoning caused by fish. Similarly, *Spiranthes diuretica* is thought to be a strong diuretic in Chile (Tzi *et al*., 2012; Bulpitt *et al.,* 2007). *Dendrobium formosum* has an amazing ability to fight tumors' (Prasad & Koch, 2014). Orchids not only serve as medicines but also provide food or used as food supplements, like *Dendrobium salaccense* leaves has been eaten with rice. Tubers of some species of Gastonia are used like a potato in some regions of Asia and orchids also provide an alternate use of glue from pseudo bulbs (Dodson, 2016). Among various cultivars of *Dendrobium*, *Queen pink* cultivar has widely been used for ornamental purpose due to its bright pink color of ever blooming flowers.

Protoplast isolation and somatic hybridization can help in plants genetic improvement, to develop new flower colors, pathogen resistance, somaclonal variation, functional genomics, genetic transformation, conservation biotechnology and pharmaceutical products. Protoplast technology is also used to produce top quality flowering ornamental plants (Musharof *et al*., 2013). In the family *Orchidaceae,* studies on protoplast started in 1978 and first isolation of protoplasts was reported in *Renentanda* from protocorms (Teo & Neumann, 1978a), from leaves of *Cattleya* and *Phalaenopsis* (Sajise & Sagawa, 1991). Seedlings of *Dendrobium* and *Paphiopedilum* were used for protoplast isolation by (Teo & Neumann 1978 b; Price & Earle, 1984) and in *Cymibidium* (Oshiro & Steinhart, 1991) protoplast of *Aranda* was isolated by (Loh & Rao, 1985; Koh *et al*., 1988; Kanchanapoom & Tongseedam, 1994). However, successful colony formation of protoplast was reported in *Dendrobium* cultivars by (Kuehnle & Nan, 1990). Successful protoplast isolation was also achieved in other ornamental plants like from callus of *Lilium longiflorum overig* by (Yousuf *et al*., 2015). Protoplast technology has been growing over time and carries great significance and attention, it has achieved great progress. Genetic manipulation through protoplast technology like somatic hybridization, cybridization or direct gene transfer can be exploited for plant improvement if a reliable and efficient plant regeneration system for isolated protoplast can be developed (Papadakis & Roublakis, 2002).

Many outstanding *Dendrobium* intrageneric and interspecific hybrid cultivars had developed from conventional breeding methods and through somaclonal variations resulting from tissue culture techniques. Somatic hybridization through protoplast fusion allows the hybridization of different sexually incompatible genera and species (Davey *et al*., 2005). The regenerative plantlets brought to existence with protoplast culture

through an efficient method and have great potential as a biotechnological tool for orchid advancement. The aim of this research was to optimize an efficient protocol for isolation of viable protoplasts from leaves of *In vitro* grown plantlets of *Queen pink* cultivar of *Dendrobium*.

Materials and Methods

Source of plant material: *Dendrobium Queen pink* pods were grown in the greenhouse of H.E.J. Biotechnology wing through artificial pollination. These pods were surface sterilized with flame and inside seeds were exposed through longitudinal cutting, and placed on MS medium. Orchid seeds were successfully germinated in growth room under 25±2^{o}C with a 16/8 hrs photoperiod. Leaves of these *In vitro* grown orchid plantlets were used as a source of explants for protoplast isolation and culture (Fig. 2A).

Explants preparation prior to enzyme exposure: *In vitro* raised leaves of *Dendrobium Queen pink* were excised and chopped transversely into fine pieces with sharp scalpel for protoplast isolation (Fig. 2B). All chopped leaves were weighed in sterilized Erlenmeyer flask under laminar flow hood for fresh weight determination. Four different measurements i.e., (0.5gm, 1.0 gm, 1.5 gm and 2.0 gm) were used to treat them with the same volume and concentration of enzyme.

Effect of Incubation time on enzyme solution: Appropriate time needed to isolate more viable protoplasts from chopped leaves of *Dendrobium Queen pink* was very important. Viable protoplast with high yield was determined with 0 hrs, 2 hrs, 4 hrs and 6 hrs incubation period.

Protoplast isolation: The protoplast isolation procedure was performed under aseptic conditions inside the laminar air flow cabinet. Protoplast isolation solution consists of cell wall digesting enzymes (1% Cellulase Onozuka R-10 (Phyto technology laboratories), 0.5% Macerozyme Onozuka R-10 (Serva) and 0.1% Pectinase Onozuka R-10 (Sigma Aldrich) along with the osmotic or washing solution with (0.3 M mannitol, 10 mM $CaCl_2.2H_2O$ and 10 mM MES, pH 5.8) which were dissolved in distilled water. The protoplast isolation solution 8ml was added to fresh weight samples of explants, then enzyme-leaves mixture was incubated on rotary shaker at 45 rpm in dark at 25ºC for 4 hours to facilitate the liberation of protoplasts (Fig. 2C).

Protoplast purification: After incubation, suspension of digested tissues in the isolation mixture was filtered through a 45µm nylon mesh sieve and the filtrate was centrifuged at 750rpm for 5min in a swing bucket centrifuge (Anke, China). The supernatant was removed using pasture pipette. The pellet of protoplasts was washed twice with washing solution through centrifugation at 750 rpm for 4mins to remove all traces of enzyme solution. Finally, the protoplast pellet was transferred to the 10ml screw capped centrifuge tube containing 4ml of 0.3M Sucrose solution and then centrifuged at 300 rpm for 4 mins. The floating viable protoplast fraction was formed at interphase of washing solution and sucrose solution, then gently removed with a pasture pipette. The purified protoplasts were then washed twice with washing solution and separated by centrifugation at 750 rpm for 4mins and resuspended in 1.5ml washing solution (Fig. 2D).

Determination of the Yield of protoplasts: The yield of the protoplast was counted using haemocytometer under a compound microscope (Nikon ECLIPSE TE 300, Japan). The protoplasts were viewed at 40× magnification and the number of protoplasts observed was recorded. Total protoplast yield was calculated using the equation below:

$$\text{Protoplast yield} = \frac{\text{Total cell count} \times \text{Total vol. of cell suspension}}{4 \times \text{Weight of fresh tissues (g)}}$$

Viability test: The protoplasts viability was examined by adding 50ul protoplasts suspended in washing solution and then mixing with 25ul FDA (Fluorescein diacetate) stock solution prepared per (Nadel, 1989). Viable cells were observed under a fluorescence microscope (Nikon TE 2000 E, Japan).

Protoplast culture for regeneration: Each sample of purified protoplasts was washed thrice with modified K&M media (Kao & Michayluk, 1975) excluding nucleic acids and amino acids except glutamine with pH adjusted to 5.7. About 2.6ml of fresh K&M medium supplemented with growth regulators 0.5 mg/l NAA, 0.4mg/l BAP and 0.5mg/l zeatin (Kunasakdakul & Smitamana, 2003) were added into each test tube. Protoplasts were taken from test tubes and transferred onto 6 cm Petri dishes. Petri plates were sealed with parafilm and incubated in dark at 25°C. The percentage of dividing protoplasts was monitored after 1 week of culture.

Results

Effect of amount of explants on yield: The chopped leaves of *Dendrobium Queen pink* gave high yield as well as good strength of viable protoplasts at the amount of 1.5gm leaves (Fig. 3C&G). In 1.5gm of leaves, protoplast yield was 15.7×10^4 protoplasts/g FW and showed high viability (Fig. 1A). On the other hand leaves with 0.5gm, 1.0gm and 2.0gm in amount resulted in low yield and poor viability as compared with 1.5gm of chopped leaves as mentioned in (Fig. 3A, B&D) and (Fig. 3E, F&H).

Effect of incubation time on yield: The best incubation time for protoplast isolation from *Dendrobium* cultivar *Queen pink in vitro* leaves was 4 hrs, at this incubation time more viable protoplasts with high yield were obtained (Fig. 4C&G). Protoplast yield in 4 hrs enzyme incubation time was 15.7×10^4 protoplasts/g FW as shown in (Fig. 1B). In 6 hrs incubation time protoplast yield was 3.6×10^4 protoplasts/g FW, large size protoplast with more debris were obtained (Fig. 4D&H). This was due to high enzymatic treatment time as more digestion of cells occurred so more debris were isolated with less viable and low yield protoplasts (Fig. 4D). After 2 hrs of incubation, very less viable cells with very low yield were obtained i.e. 1.0×10^4 protoplasts/g FW (Fig. 4B&F).

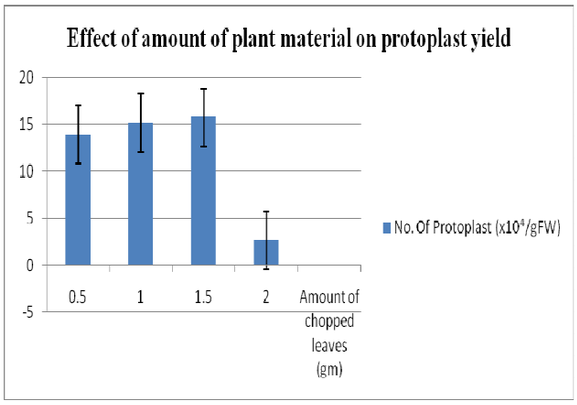

Fig. 1A. Effects of different amount of explants on protoplast isolation of *Dendrobium Queen pink*.

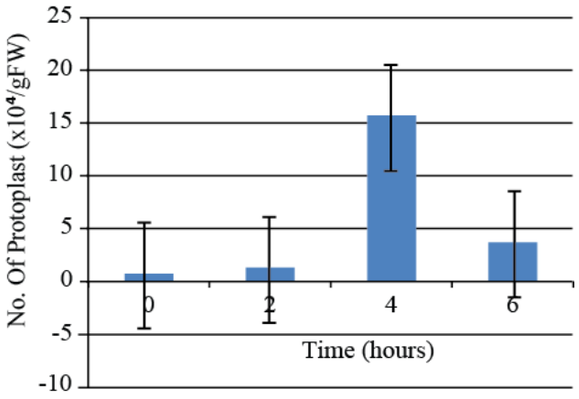

Fig. 1B. Effect of incubation time on protoplast isolation of *Dendrobium Queen pink.*

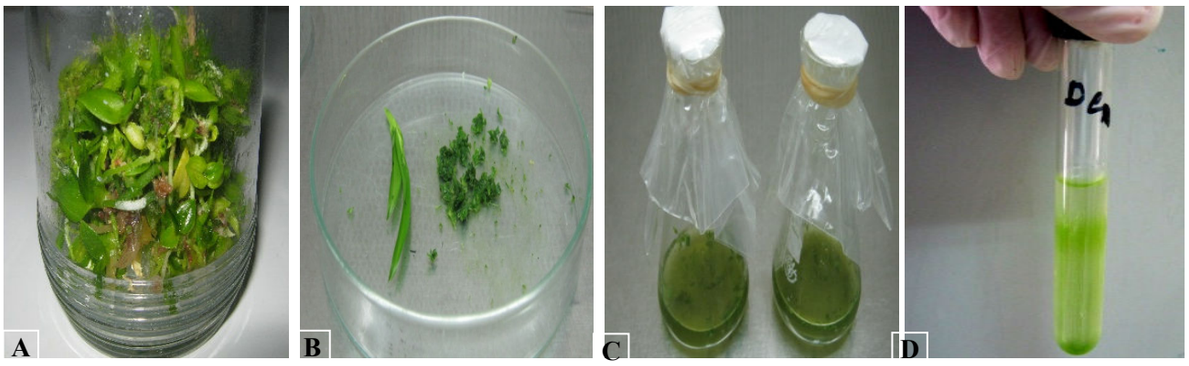

Fig. 2. **A**. *In vitro* grown plantlets of *Dendrobium* cultivar *Queen pink.* **B**. Chopped leaves. **C**. Incubated chopped leaves in enzyme solution. **D**. Isolated protoplasts in a test tube.

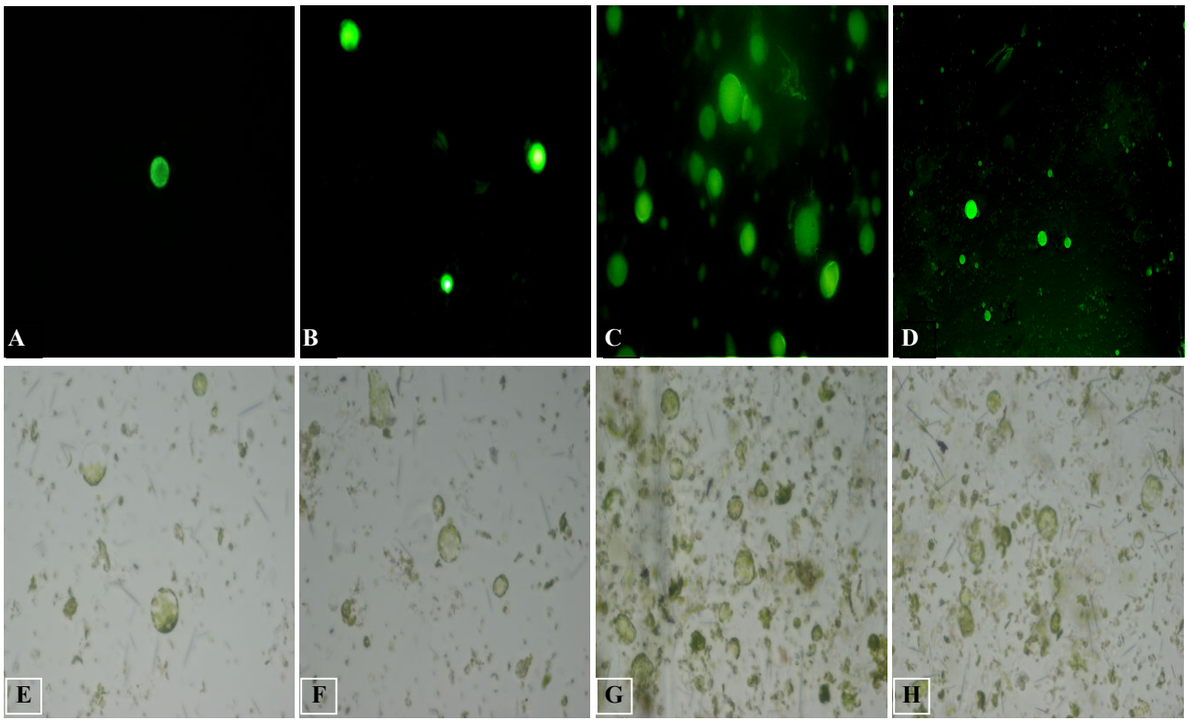

Fig. 3. **A**. Protoplast viability in 0.5gms of leaves. **B**. Protoplast viability in 1.0gms of leaves. **C**. Protoplast viability in 1.5gms of leaves. **D**. Protoplast viability in 2.0gms of leaves. **E**. Protoplast yield in 0.5gms leaves under bright field. **F**. Protoplast yield in 1.0gms of leaves under bright field. **G**. Protoplast yield in 1.5gms of leaves under bright field. **H**. Protoplast yield in 2gms of leaves under bright field.

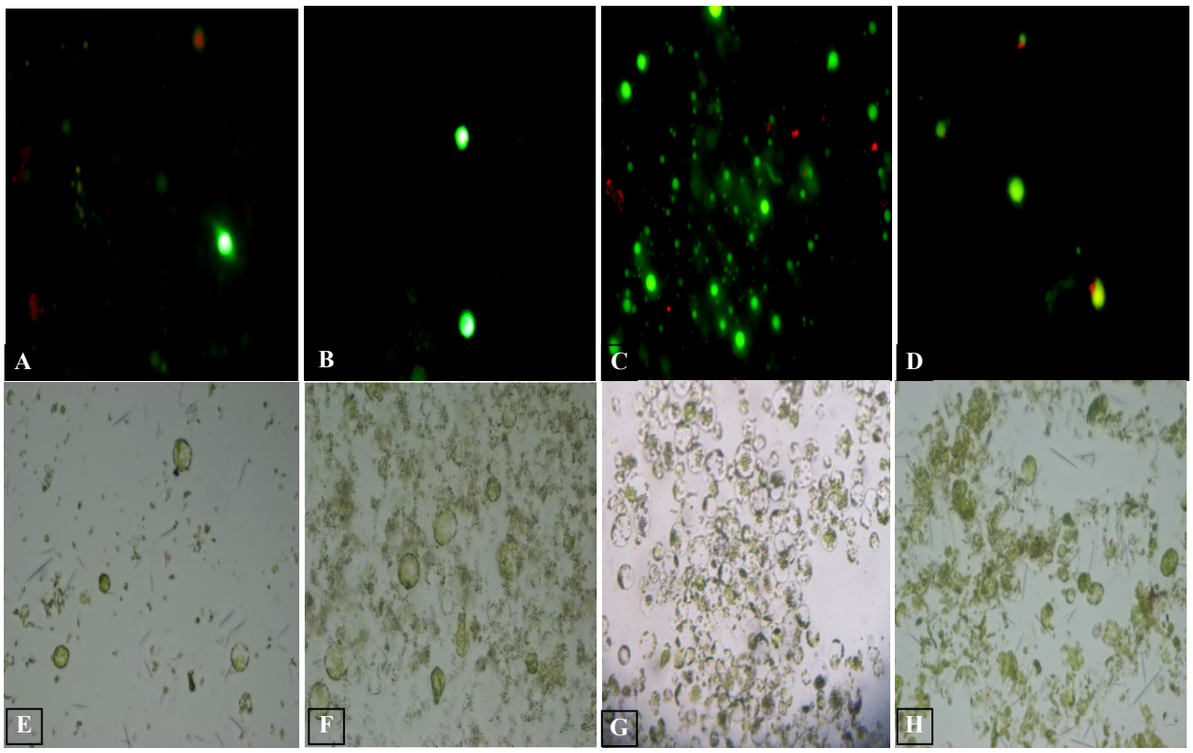

Fig. 4. **A**. 0hrs yield of viable protoplasts. **B**. 2hrs yield of viable protoplasts. **C**. 4hrs yield of viable protoplasts. **D**. 6hrs yield of viable protoplasts. **E**. 0hrs yield of protoplast under bright field. **F**. 2hrs yield of protoplasts under bright field. **G**. 4hrs yield of protoplasts under bright field. **H**.6hrs yield of protoplasts under bright field.

Discussion

(a) Effect of amount of explants on protoplast yield and viability: The successful isolation of protoplasts depends on many factors; it is also related to the source plant and the conditions of the applied methods. The isolation conditions are extremely important for the release of protoplasts, without losing cell viability and to get maximum yield. The result of this study on *Dendrobium* cultivar *Queen pink* demonstrated that the protoplast yield and viability also influenced by the amount of explants i.e., leaves used. According to (Pindel, 2007) mesophyll tissues of *cymbidium* are good source material for protoplast isolation and culture.

We used different amount of explants i.e., 0.5gm, 1.0gm, 1.5gm and 2.0gm leaves of *In vitro* grown *Dendrobium* cultivar *Queen pink* plantlets (Fig. 2). Among these, the highest yield of 15.7×10^4 protoplasts/g FW with greater viability was achieved when leaves of 1.5g were used as shown in (Fig. 3C&G). (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.

The freshly isolated protoplasts were of spherical shapes with different sizes and appeared yellow green in FDA staining. In addition, protoplasts derived from Dendrobium mesophyll cells were stable after staining (Fig. 3). According to (Pindel, 2007) 0.3g of *Cymbidium* was incubated in 10 ml enzyme solution (1.2% macerozyme and 0.5% pectinase) they had given highest yield and viability. Similarly 0.3g leaves of *Dendrobium pompadour* was incubated in 5ml enzyme solution (1% cellulase, 0.5% driselase, 1% macerozyme) provided best yield of viable protoplasts reported by (Kanchanapoom *et al*., 2001). Also highest viable protoplast yield was achieved when 0.5g leaves of *Dendrobium crumenatum* were used (Chong *et al*., 2010). (Yuphin *et al*., 2006) reported that 1.0g leaves of *Dendrobium sonia* gave highest viability as well as yield.

(b) Effects of incubation time on protoplast yield and viability: To determine the suitable duration required for obtaining the highest yield of protoplasts, the explants were incubated with enzymes for different incubation periods like 0, 2, 4 and 6 hrs (Fig. 4). The low yield of viable protoplasts was achieved after 0 hr and 2 hrs of incubation; protoplasts were smaller in size and oval in shape (Fig. 4A&B). In 0 hrs and 2 hrs incubation time cell wall was removed, it might be due to osmotic stress because this was insufficient time for enzymes to release large number of protoplasts (Fig. 4E&F).

The yield of protoplasts was increased when the incubation time extends to 4 hrs which is 15.7×10^4 protoplasts/g FW (Fig. 1B). Protoplasts appeared in proper shape and size and the organelles had seen to be aggregated at a point within the membrane (Fig. 4G). When incubation time was increased up to 6 hrs, yield and viability of protoplasts decreased and protoplasts were observed large in size with more debris, this is because 6 hrs was more than enough time for enzymes to digest whole cell wall (Fig. 4H), it caused protoplasts to burst.

In this study, the suitable incubation time for isolation of protoplasts from leaves of *In vitro* grown plantlets of *Queen pink* was 4 hrs, because 4 hrs provided enough time for the enzymes to release large number of protoplasts (Fig. 4C).

These findings are in agreement with the work of (Chong *et al*., 2010; Yuphin *et al*., 2006) that optimum time for protoplast isolation from *Dendrobium In vitro* leaves is 4 hrs protoplast yield was decreased in 6 hrs. Present results are in contrast with the result of (Kanchanapoom *et al*., 2001) who reported that 3 hrs was the best incubation time for protoplast isolation from mesophyll cells of *Dendrobium Pompadoue*. According to (Prasertsongskun, 2004), protoplasts were isolated efficiently from explants by 10 hrs incubation, with the enzyme mixture containing 2% cellulase, 2% macerozyme and 0.5% pectinase. This study is in disagreement with the results of (Shrestha & Tokuhara, 2007) that protoplasts derived from the callus of *Phalaenopsis* were disrupted after 7 hrs incubation times.

Conclusion

The isolation conditions are extremely important for the efficient release of protoplasts from *In vitro* leaves of *Queen pink* cultivar of *Dendrobium.* Based on the results 1.5gm leaves and 4 hrs incubation periods were found the most suitable conditions for protoplast isolation. The established protocol could be used to explore the possibility to create inter generic somatic hybrids of orchids by protoplast culture technology as well as for future research in manipulating the genes, particularly in protoplast fusion study.

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References

Bulpitt, C.H., L. Yan, P.F. Bulpitt and J. Wang. 2007. The use of orchids in Chinese medicines. *JRSM*., 100(12): 558-563.

Chong, S.T., S.L. Pui, P.K. Anna Ling and M. Maziah. 2010. Optimization of protoplast isolation protocols using in vitro leaves of *Dendrobium crumentum* (pigeon orchid). *Afr. J. Agric. Res.,* 5(9): 2685-2693.

Davey, A.R., P. Anthony, J.B. Power and K.C. Lowe. 2005. Plant protoplasts: status and biotechnological perspectives. *Biotech. Adv.*, 23: 131-171.

Dodson, C.H. 2016. Orchids. Encyclopaedia Britanncia. http://www.britannica.com/EBchecked/topic/431285/orchid/73081/Economic-importance.

Dodson, C.H. 2016. Orchid plants. Encyclopaedia Britannica. http://www.britannica.com/plant/orchid.

Kanchanapoom, K. and P. Tongseedam. 1994. Conditions for isolation culture of Arenda Chark Kuan mesophyll protoplasts. *J. Sci. Soc. Thai.*, 20: 63-72.

Kanchanapoom, K., J. Saowarath and R. Daungkae. 2001. Isolation and Fusion of Protoplast from Mesophyll cells of *Dendrobium Pompadoue. Sci. Asia..,* 27: 29-34.

Kao, K.N. and M.R. Michayluk. 1975. Nutritional requirements for growth of *Vicaia hajastana* cells and protoplasts at a very low population density in liquid media. *Planta*., 126: 105-110.

Koh, M.C., C.J. Goh and C.S. Loh. 1988. Protoplast isolation and culture of Aranda hybrids. *Malayan orchid Rev.,* 22: 70-78.

Kuehnle, A.R. and G.L. Nan. 1990. Factors influencing the isolation and culture of protoplasts from Hawaiian Dendrobium cultivars. In: (Eds.): Bonham, D.G. & J. Kernohan. *Proceedings of the 13th World Orchid conference, Auckland, New Zealand*. pp. 259-262.

Kunasakdakul, K. and P. Smitamana. 2003. Dendrobium prathum red protoplast culture. *Thai J. Of Agric. Sci*., 36: 1-3.

Loh, C.H. and A.N. Rao. 1985. Isolation and culture of meophyll protoplast of Aranda Noorah Alsagoff. *Malayan Orchid Rev*., 19: 34-37.

Musharof, M.H., K. Ravi, T.V. Pham, B. Winarto, Z. Songjun, A. Jaime and S. Teixeira. 2013. The application of biotechnology to orchids. *Critical Reviews in Plant Sciences*, 32(2): 69-139.

Nadel, B.L. 1989. Use of fluorescein diacetate in citrus tissue culture for determination of cell viability and the selection of mutants. *Scientia Horticulturae*., 39: 15-21.

Oshiro, M.A. and W.L. Steinhart. 1991. Preparation of Protoplasts from cells of orchids representing various genra. *Lindleyana*., 6(1): 36-41.

Papadakis, A.K. and A. Roubelakis. 2002. Oxidative stress could be responsible for the recalcitrance of plant protoplast. *Plant physiol. Biochem.,* 40: 549-559.

Pindel, A. 2007. Optimization of isolation conditions of *Cymbidium* protoplasts. *Folia Horticulturae. Ann.,* 19(2): 79-88.

Prasad, R. and B. Koch. 2014. Antitumor activity of ethanolic extract of *Dendrobium formosum* in T-Cell lymphoma: An *In vitro* and *In vivo* study. *Biomed Res Int*., 1-11.

Prasertsongskun, S. 2004. Isolation and culture of suspension protoplasts of vetiver. *J. Sci. Technol.,* 26(3): 411-416.

Price, G.R. and E.D. Earle. 1984. Sources of orchid protoplasts for fusion experiments. *Amer Orchid Soc Bull*., 53: 1035-1043.

Robert, L.D. and D. Calaway. 1960. Classification and phylogeny in the Orchidaceae. *Annals of the Missouri Botanical Garden*, 47(1): 25-68.

Sajise, J.U. and Y. Sagawa. 1991. Regeneration of plantlets from callus and protoplasts of *Phalaenopsis* sp. *Malayan Orchid Bull*., 5: 23-28.

Shrestha. B.R. and M. Tokuhara. 2007. Plant Regeneration from cell suspension-derived Protoplasts of Phelonopsis. *Plant cell Rep.,* 26(7): 719-725.

Teo, C.H.K. and K.H. Neumann. 1978. The culture of protoplasts isolated from Renantenda Rosalind Cheok. *Orchid Rev.*, 86(2): 156-158.

Teo, C.K.H. and K.H. Neumann. 1978. The isolation and hybridization of protoplast from orchids. *Orchid Rev.*, 86: 186-189.

Tzi, B., L. Jingyi, J.H. Wong, Y. Xiujuan, S.C. Wing Sze, T. Yao and Y.Z. Kalin. 2012. Review of research on *Dendrobium*, a prized folk medicine. *App. Micro. & Biotech.,* 93(5): 1795-1803.

Yuphin, K., P. Ampaiwan, T. Sureeya, P. Salak and T. Niphone. 2006. Protoplast isolation and culture of *Dendrobium Sonia* ``Bom 17``. *Kasetsart J. (Nat. Sci.).,* 40: 361-369.

Yousuf, S., F. Ashraf, S.K. Kazmi, S. Khan and H.A. Kayani. 2015. A study on the isolation of protoplasts from the callus of *Lilium longiflorum* overig. *Pak. J. Bot*., 47(6): 2391-2396.

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