

## Optimization of Protoplast Isolation Methods from Leaves in *Chrysanthemum morifolium* 'Jinba'

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### ABSTRACT

*Chrysanthemum morifolium* 'Jinba' (Asteraceae) is one of the most important standard-type cultivars and cut flower species. The use of chrysanthemum protoplast is the need for transgenic, mutagenic breeding, and CRISPR/Cas9 programs of this species. The conduct of protoplast isolation has been continuously maintained in *C. morifolium* 'Jinba' for its claim. In this study, the incubation time, mannitol, and cellulase concentration were reported to optimize the efficient condition for the chrysanthemum protoplast. The results showed the high yield of protoplast was isolated at 0.6 M mannitol concentration, 1% cellulase, and at 3 hours incubation time in the enzyme combination with 0.3% macerozyme and 1% pectinase.

**Keywords:** Cellulose, Chrysanthemum, Incubation time, Mannitol, Protoplast isolation.

### INTRODUCTION

The use of protoplast is the most considered as an extensive and beneficial approach to contribute to *in vitro* genetic engineering, metabolites compounds, hybridization, especially in CRISPR/Cas9 transgenic methods [1-5]. The Asteraceae species contain a lot of floricultural and ornamental cultivars and wild types, including chrysanthemum, which is more interesting in medicinal and pharmacological interest, and is one of the economic global pot and cut flower, and is one of the gigantic genome for studying in gene function, genetic variation, and heredity in Chrysanthemum breeding [6,7].

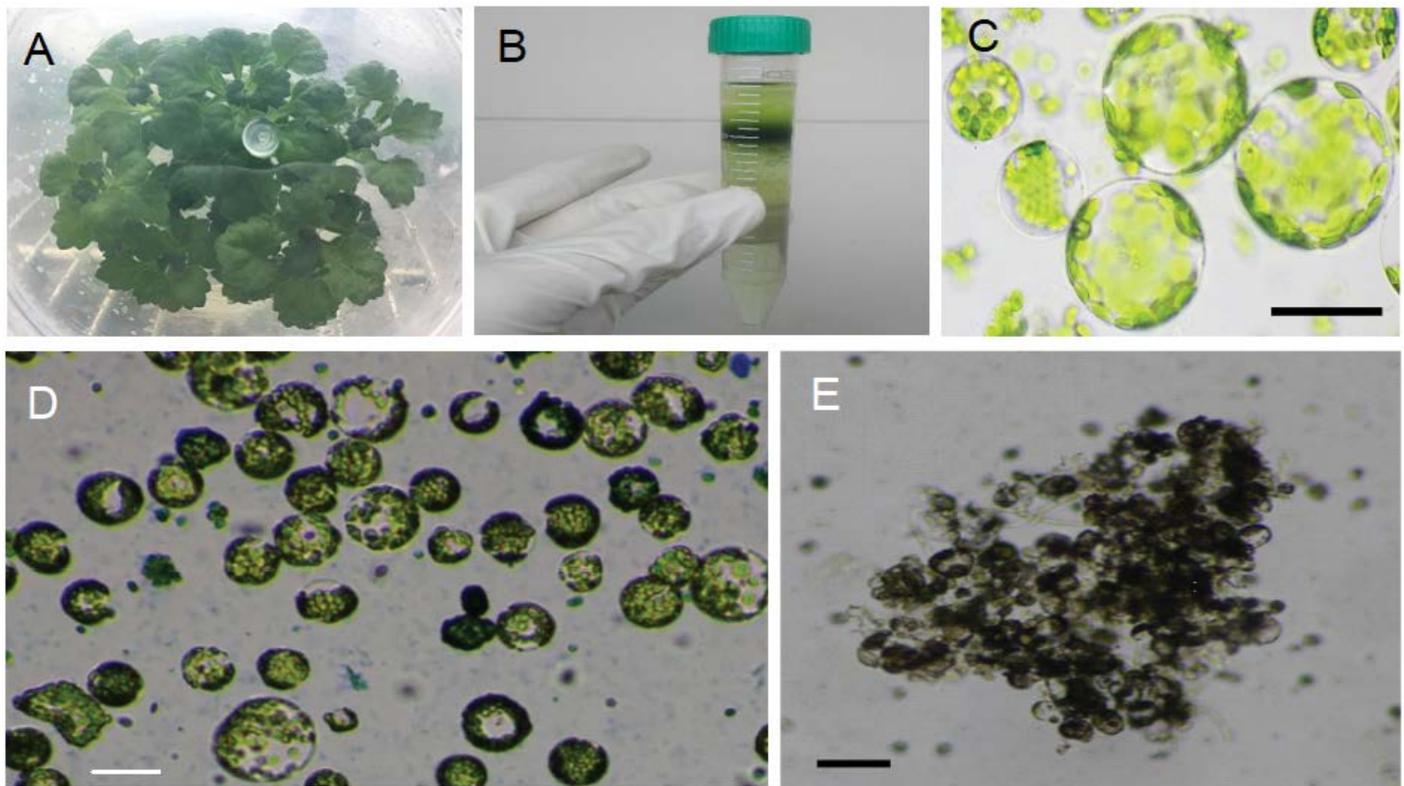
There are several reports in the conditional methods for chrysanthemum mesophyll protoplast, however, there is still a several-considering improvement of each cultivar in the regeneration and efficiency from protoplast to plant in the achievements of individual cultivar [8-11].

In-plant culture technology, the protoplast technic has been developed and confirmed to produce the percentage of cell division and optimize the chemical composition in the protoplast culture medium. In this study, we also compared and reported the effect of the influence of culture conditions, such as incubation time, mannitol, and cellulase concentration to optimize the efficient-objective chrysanthemum protoplast to conduct the design of first step protoplast isolation in standard chrysanthemum cultivar *C. morifolium* 'Jinba'.

### MATERIALS AND METHODS

Plant stock cultures of *C. morifolium* 'Jinba' were grown in Phytohealth container (103 × 78.6 mm, SPL Life Sciences, Korea) at 25 ± 1°C under a 16 h photoperiod at 30-40 μmol m<sup>-2</sup>s<sup>-1</sup> photosynthetic photon radiation provided by cool-white fluorescent lamps (Kumho FL40EX-D, Korea) (Figure 1A). After 4 weeks, the young leaves were chopped into small pieces into mannitol for 1 h to correspond with the mannitol concentration in the protoplast isolation solution. They were consequently incubated on a shaking machine (40 rpm) in the enzyme solutions containing the different concentration of Cellulase Onozuka R-10 (Yakult Pharmaceutical Ind. Co., LTD., Japan), 0.3% Macerozyme R-10 (Yakult Pharmaceutical Ind. Co., LTD., Japan), and 1% Pectinase from *Aspergillus niger* (Sigma-Aldrich, USA) dissolved in MS medium (pH 5.8) with mannitol concentration as the same concentration of the pre-treatment. The different concentration of mannitol (0.4 M, 0.5 M, 0.6 M, and 0.7 M), the different concentration of cellulase (0.5%, 0.8%, 1.0%, and 1.5%), and the different incubation time (2 h, 3 h, 4 h, and 5 h) were evaluated in the dark condition in this study. After incubation, the mixture of protoplast and enzyme solution was filtered by cell trainer (100 μm, SPL Life Sciences, Korea). After centrifugation (500

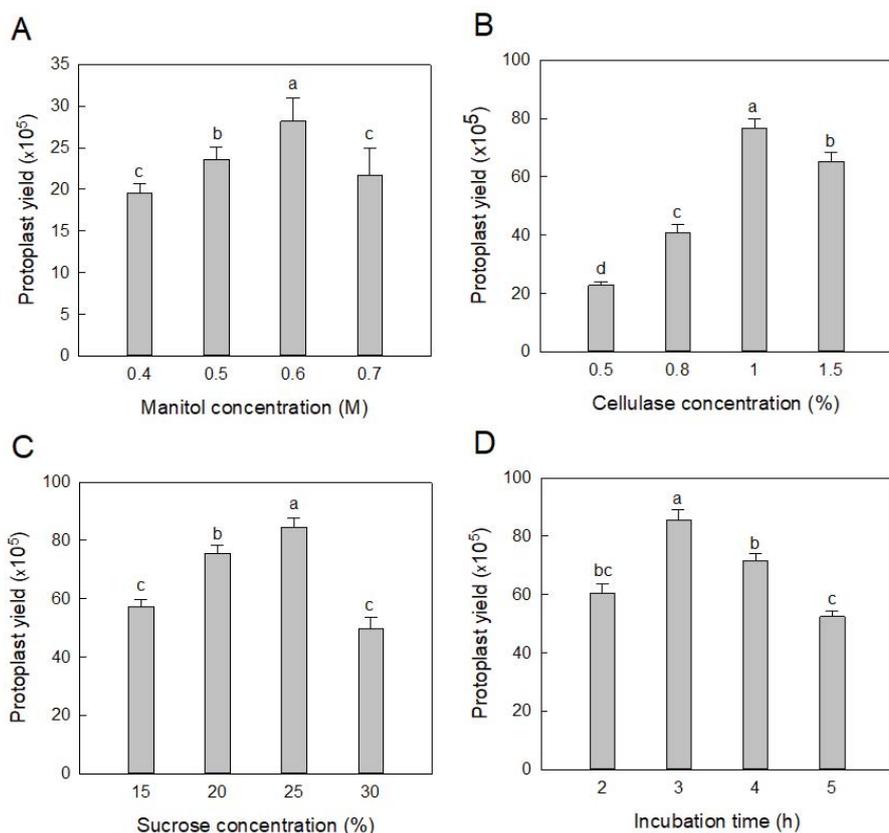
rpm, 10 min) in a conical tube (50 ml, 50050, SPL Life Sciences, Korea), the pellet was resuspended in washing medium containing the same mannitol concentration with pre-treatment and MS medium (pH 5.8). The protoplasts were classified by density-gradient centrifugation with the different sucrose concentrations (15%, 20%, 25%, and 30%) (Figure 1B). After four centrifugation and washing times, the protoplast concentration was counted in a disposable hemocytometer (C-Chip, INCYTO, Korea) and evaluated viability by Evans blue staining method (Figure 1D). Protoplast culture was started at a concentration of  $2.5 \times 10^4$  protoplasts per milliliter with 10 ml protoplast culture medium containing KM vitamin with 1 mg/l BAP, 2 mg/l NAA, and 1 mg/l 2, 4-D in 5 cm diameter petri dish [11]. Data were achieved by variance analysis (ANOVA) using Statistical Product and Service Solutions (SPSS Version 18.0) with significant differences at  $p < 0.05$  based on Duncan's multiple range tests.



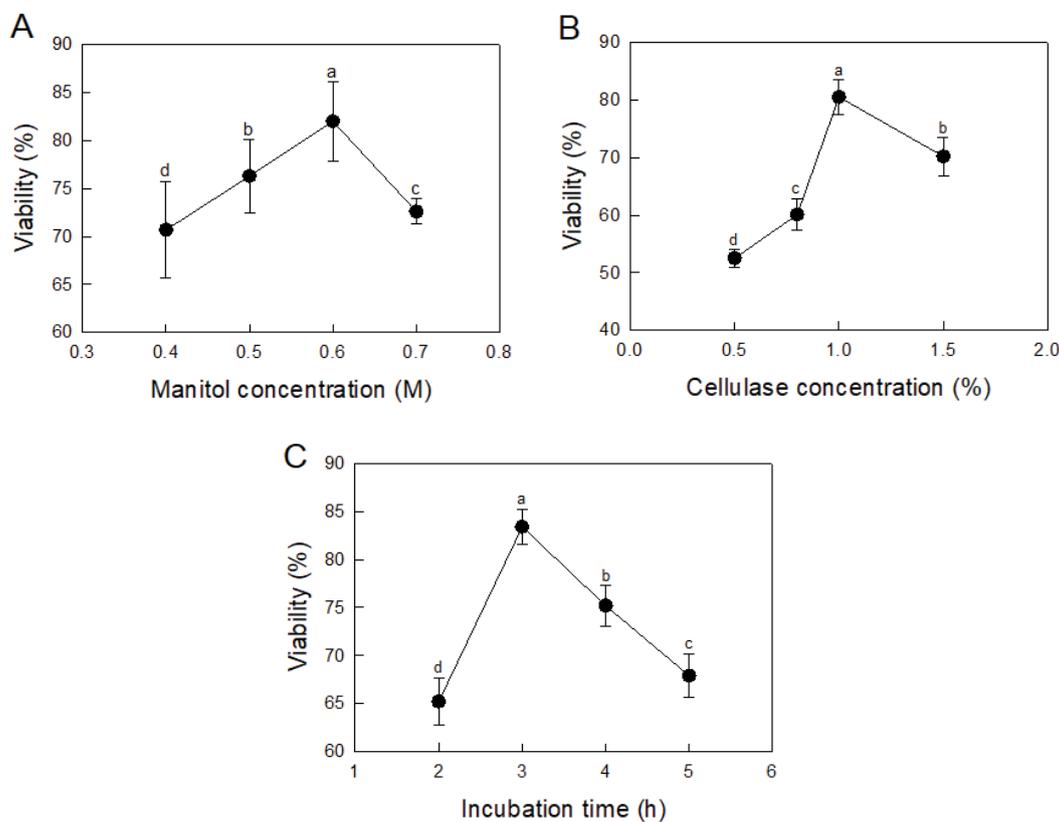
**Figure 1:** Isolation and regeneration of protoplasts from *in vitro* leaves of *Chrysanthemum morifolium* 'Jinba'. A): four-week-old *in vitro* chrysanthemum; B): density-gradient centrifugation; C): freshly isolated mesophyll protoplasts, bar=20  $\mu$ m; D): freshly isolated mesophyll protoplasts stained with Evan's blue dye, bar=30  $\mu$ m; and E, protoplast-derived microcalli, bar=100  $\mu$ m.

## RESULTS AND DISCUSSIONS

*Chrysanthemum* protoplast was collected from the leaf pieces after incubation with enzyme combinations of Cellulase R-10, Macerozyme R-10, and Pectinase (Figure 1C). The protoplast-derived microcalli were developed in 4 weeks after protoplast isolation (Figure 1E). Based on the results displayed in Figure 2, the experiments can be concluded that the difference in the protoplast isolated yield is caused by the difference in mannitol and cellulase concentration, incubation time, and sucrose concentration. The protoplast yield was achieved by mannitol concentration (maximum in 0.6 M), cellulase concentration (approximate at 1%), incubation time (effective in 3 hr), and sucrose concentration for density-gradient centrifugation (performed in 25%) (Figure 2). The viability of protoplast was evaluated by Evans blue staining method according to mannitol concentration (maximum in 0.6 M), cellulase concentration (highest at 1.0%), and incubation time (best in 3 hr). Using more mannitol and more enzyme should not be harmful to the *chrysanthemum* protoplasts, the cell wall is degradable by three enzymes to affect only cell component. However, the higher mannitol concentration (0.7 M) and cellulase concentration (1.5%) were shown a decrease in protoplast yield. It seems the osmotic and turgor pressures in physiological problems between water loss and water absorption in plants [12]. On the other hand, the high enzyme levels are used to set up the short incubation times are considered [13], the most effective incubation time is 3 hr in this study.



**Figure 2:** (A): Effects of mannitol concentration; (B): cellulase concentration; (C): sucrose concentration and; (D): incubation time on protoplast yield. All values are presented as means  $\pm$  SE (n=5). Different letters (a-d) among treatments indicate statically significant differences at  $p < 0.05$  based on Duncan's multiple range test.



**Figure 3:** (A): Effects of mannitol concentration; (B): cellulase concentration and; (C): incubation time on protoplast viability. All values are presented as means  $\pm$  SE (n=5). Different letters (a-d) among treatments indicate statically significant differences at  $p < 0.05$  based on Duncan's multiple range test.

Using the high concentration of mannitol and isolated enzyme within a long incubation time, it was not intended to be used for achieving a high number of protoplast yield. The researcher should be considered between the isolated cost (the cost of enzymes) and the high number of protoplasts. The purification of protoplast may be a consequence of the difference in protoplast originating from different tissue and protoplast density [14]. In this study, the use of 25% sucrose was shown the highest of protoplast yield. The effects on the isolation method should be prioritized over maximizing the protoplast yield. The viability of protoplast was evaluated and shown that the proper isolated method should be with 0.6 M mannitol, 1% cellulase, and 3 hr incubation in *C. morifolium* 'Jinba' (Figure 3). All of the protoplasts were achieved by the proper isolated method to have the opportunities to develop into protoplast-derived microcalli within 4 weeks after isolation (Figure 1E).

### CONCLUSION

In this study, an accessible and proper isolated method has been established for chrysanthemum protoplasts in the standard cultivar. There are several factors were optimized for getting the highest viability and protoplast yield such as mannitol, cellulase concentration, incubation time, and the different sucrose concentration in density-gradient centrifugation. This study is not only providing the experimental protoplast isolation but also focusing on the demonstration of the approach to several factors into chrysanthemum protoplast culture systems as further studies.

### ACKNOWLEDGEMENT

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