

Effect of Different Cellulase and Pectinase Enzyme Treatments on Protoplast Isolation and Viability in *Lilium ledebourii* Boiss.

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Abstract

For overcoming interspecific incompatibility, protoplast combination method is a proper procedure for making a new plant with desired traits. For this purpose, protoplast preparation is a first and important step. Hence, experiments were conducted to evaluate various combinations of cellulase, pectinase and their treatment times on protoplast production and protoplast viability in *Lilium ledebourii* Boiss. The results of experiment revealed that the protoplast yield was significantly affected by different treatment levels. Cellulase at 4% gave the highest numbers of protoplasts at 3.71×10^5 protoplast/g FW. Pectinase at 1% gave the highest numbers of protoplast. For treatment times, the highest yield of protoplast was with leaf explants treated for 24 h. Analysis of variance indicated that concentration, time and three-way interaction of cellulase, pectinase and time were significant at $p < 0.01$. Cellulase at 4% and pectinase at 0.2% for 24 h gave the highest viability. Interactions of cellulase \times pectinase, cellulase \times time, pectinase \times time and cellulase \times pectinase \times treatment time were significant at $P \leq 0.05$ for protoplast number. The highest and lowest protoplast numbers were produced in media containing 4% cellulase and 1% pectinase for 24 h (6.65×10^5 protoplast/g FW) and 1% cellulase and 0.2% pectinase for 12 h, respectively. It's concluded that, the best treatment for isolation of *Lilium* protoplast was 4% cellulase and 1% pectinase for 24 h.

Keywords: cellulase, *Lilium ledebourii* Boiss., pectinase, protoplast isolation, viability

Introduction

Lily flowers are admired and cultivated throughout the world. The conventional breeding of lily species has provided novel cultivars with resistance to viral diseases, tolerance for unfavorable culture conditions, and a variety of flower colours and forms (Van Tuyl *et al.*, 1988).

Lilium ledebourii (Baker) Boiss. (Family Liliaceae) is called 'Susan-e-chelcheragh' in Persian and is the only endangered species of the *Lilium* genus in Iran. It has considered potential as an ornamental plant due to its large and attractive white flowers equal to those of commercial lilies (Wendelbo, 1977). *L. ledebourii* can be used for breeding programs with its large raceme of 2-15 flowers. The number of plants of this species is continuously decreasing in nature because of cutting the plants and removing underground organs (Khosh-khui and Azadis, 2007). The application of *in-vitro* propagation techniques can offer the possibility of producing large number of uniform plants for breeding programs and field cultivation.

Protoplasts are isolated primarily by mechanical or enzymatic methods. Mechanical isolation is used only occasionally, but remains historically important. Large

quantities of protoplasts can potentially be obtained with enzymatic methods. Less breakage and much less osmotic shrinkage occurs compared with mechanical methods (Torres, 1988). The success of protoplasts isolation depends on the condition of the tissue and the combination of enzymes being used (Galbraith *et al.*, 1984; Rasheed *et al.*, 1990; Saker *et al.*, 1999). Effects of cell densities for protoplast development have also been reported (Kuchuk *et al.*, 1998; Saito and Suzuki, 1999).

Several factors influence protoplast release, including the extent of cell wall thickening, temperature, duration of enzyme incubation, pH of the enzyme solution (Sinha *et al.*, 2003), agitation, the nature of the osmoticum, and plasmolysis prior to enzyme digestion of source tissues in salts (Frearson, 1973). Protoplast yield and viability can be enhanced by slicing of source pre-plasmolysed tissues, manual or enzymatic removal of the epidermis, and conditioning of the donor material or its culture on media containing suitable osmoticum (Davey *et al.*, 2004). The development of protoplast based systems has increased the utility of plants in biochemical and genetic research (Rao and Prakash, 1995), as well as providing greater prospects in genetic improvement of medicinal plants (Azad *et al.*,

2006). Technology and regeneration procedures play an increasingly significant role in plant improvement through somatic hybridization and protoplast transformation (Umate *et al.*, 2005). Protoplasts have served as recipient hosts for DNA transformation and are required in somatic hybridization by protoplasts fusion (Stephen, 1995).

A first step towards the plant genetic manipulation and an integrated breeding programs is an efficient protocol for protoplast isolation, culture and regeneration (Duquenne *et al.*, 2007). Leaf mesophyll cells of a wide range of plants have been used with success as a protoplast source (Saker *et al.*, 1999). Protoplasts have been isolated from various genotypes of *Petunia × hybrida* (Izhar and Power, 1977; Vasil and Vasil, 1974), *P. parodii*, (Hayward and Power, 1975), *P. inflata*, *P. violacea* and *P. axillaris* (Dulieu *et al.*, 1983; Power *et al.*, 1976). Sink and Power (1977) reported that protoplasts of the apical region including leaf primordia proved to be superior to leaves for protoplasts isolation.

There are no published reports on the isolation and culturing of protoplasts from *Lilium ledebeourii* (Baker) Boiss. The objective of this study was to determine a protocol for isolation and culturing of protoplasts from *Lilium ledebeourii* (Baker) Boiss.

Materials and methods

Leaves of *L. ledebeourii* were used as the protoplast sources in this study. Leaf explants were cut into small pieces 1 cm in length and digested with 6 different enzyme solutions: (1) 4% cellulase Onozuka R-10, 1% pectinase; (2) 4% cellulase Onozuka R10, 0.2% pectinase; (3) 2% cellulase Onozuka R-10, 1% Pectinase; (4) 2% cellulose Onozuka R-10, 0.2% pectinase; (5) 1% cellulase Onozuka R-10, 1% pectinase, and (6) 1% cellulase Onozuka R10, 0.2% pectinase. The enzymes were dissolved in cell protoplast washing (CPW) salt solution containing 13% (w/v) mannitol. The pH of the enzyme solutions was adjusted to 5.8 with 0.2N KOH or 1N HCL. Before enzyme treatment, the explants were placed with their exposed mesophyll and palisade tissues onto 30 ml of cell protoplast washing (CPW) with 13% mannitol solution in a 14 cm diameter Petri dish. Enzyme solutions were filter-sterilised through 0.2 µm membrane filters (Milipore High-Flow, Sartorius, Germany). The dishes containing leaves and

enzymes were sealed with Parafilm™ and incubated at 70 rpm for either 12 or 24 h on a rotary shaker in the dark at 25±2°C. For purification, digested leaf and enzyme solutions were filtered through sterile 80 µm mesh nylon sieve (Wilson Sieves, Nottingham, UK) to remove coarse and undigested materials. The collected enzyme with protoplasts was transferred to 15 ml capacity screw-capped centrifuge tubes (Corning Ltd., New York, USA) and centrifuged at x 300 g for 10 min. The pellet was re-suspended in washing solution the same as with enzyme solution but without the enzymes and then centrifuge twice at 300 g for 10 min. Flotation purification was carried out with 21% sucrose at x 100 g for 5 minutes. Yields of protoplasts were determined using a double-chamber haemocytometer (Modified-Fuchs Rosenthal rulings, model B.S. 74B; Weber Scientific Internaional Ltd., Teddington, UK). The viability of protoplasts was assessed by uptake and cleavage of Trypan blue such that vital protoplasts did not show uptake. Counts of viable protoplasts were made from at least 4 fields of view from each slide and the proportion (%) of viable protoplasts calculated. Each treatment was carried out in 3 replicates and the experiment was repeated twice. Data were analyzed using SPSS (ANOVA) Version 16.0.

Results and discussion

Analysis of variance (ANOVA) showed significant differences among different levels of cellulase, pectinase and treatment times. Using 4% cellulase produced the highest number of protoplasts, the average being 3.71×10^5 protoplast/gFW (Tab. 1). Among pectinase treatments, 1% produced the highest number of protoplasts. In the case of treatment time, the highest number of protoplasts was for leaf explants treated for 24 h. The ANOVA revealed interaction effects at $p \leq 0.05$ of cellulase × pectinase, cellulase × time, pectinase × time and cellulase × pectinase × time on protoplast number. Means comparison by Duncan's Multiple Range Test (DMRT) revealed that the highest and lowest protoplast numbers were produced in media containing 4% cellulase and 1% pectinase for 24 h (6.65×10^5 protoplast/g FW) and 1% cellulase and 0.2% pectinase for 12 h (9.00×10^4 protoplast/g FW), respectively. Thus, the best treatment for isolation of *Lilium* protoplasts was 4%

Tab. 1. Analysis of variance on numbers of isolated protoplasts (protoplast/ml) from *Lilium ledeoborri*

Source of variation	Df	MS	F	p value
Cellulase	2	1.877×10^{11}	278.47	0.000
Pectinase	1	2.091×10^{11}	310.26	0.000
Time	1	1.053×10^{11}	156.237	0.000
Cellulase×Pectinase	2	3.675×10^{10}	54.523	0.000
Cellulase×time	2	4.547×10^{10}	67.463	0.000
Pectinase×time	1	1.619×10^{11}	24.025	0.000
Cellulase×Pectinase×time	2	2.692×10^{10}	39.937	0.000
Error	36	6.741×10^8		

cellulase plus 1% pectinase for the 24 h treatment time. The efficiency of protoplast isolation and culture depends on many factors, such as the enzyme mixture, the presence of growth regulator and the length of time after subculture (Assani *et al.*, 2001). This result is similar to that of Nagao (1982) who reported that 2% cellulase plus 5% macerozyme and 0.5% mannitol at 25-20°C in the dark for 12-18 hr gave high isolation of protoplasts (Tab. 2). On the other hand, enzyme solution containing 4% (w/v) cellulase (Onozuka RS), 0.5% (w/v) Macerozyme R-10, both from Yakult Honsha (Tokyo), and 0.1% (w/v) pectolyase Y-23, from Seishin Pharmaceutical (Tokyo), dissolved in a washing solution of 0.9 M sorbitol, 10 mM CaCl₂·2H₂O and 5 mM MES (2-morpholino-ethanesulfonic acid) at pH 5.6 and incubated statically under dark conditions for 2 h at room temperature gave a high protoplast yield with the oriental hybrid lily cvs. 'Casablanca', 'Siberia', and 'Acapulco'. The optimum combination of enzymes for protoplast isolation from *Hypericum perforatum* L. was 14% cellulase + 1.5 % pectinase + 1% macerozyme, + 0.5 % driselase (Saker *et al.*, 1999). Nagata and Takede (1984) succeeded in isolating of plant protoplasts from *Nicotiana tobacum* L. leaves using enzymes solution for maceration of leaves containing 0.1% pectolyase Y-23 and 0.5% potassium dextran sulfate dissolved in 0.6 M mannitol solution, and the pH was adjusted to 5.8 with 0.1 N HCL (Fig. 1). Then, 1% cellulase solution which was dissolved in 0.6 M manitol solution at pH=5.2, was added to the enzyme solution. Also, they mentioned that this procedure yielded about 10⁷ protoplasts of palisade cells from 1 g fresh weight tobacco leaves. The optimum condition for protoplast isolation from vetiver was established by using 2% cellulase (Onozuka R10), 2% macerozyme R10, 0.5% pectinase in 0.4 M mannitol and 7 mM CaCl₂·2H₂O at pH 5.8 and incubated for 10 hours in the dark on the rotary shaker at 50 rpm (Prasertsongskun, 2004). Karim and Adachi (1997) reported the successful isolation and culture of protoplast of *Allium cepa* from cell suspension culture. The ANOVA for viability indicated effects at $p < 0.01$ for cellulase and pectinase concentration, time and the interaction. Means comparison for protoplast viability showed that the treatment combinations of 4% cellulase, 0.2% pectinase and 24 h and 2% cellulase, 1% pectinase and 24 h gave the highest and lowest protoplast viabilities, respectively, as compared to all other treatments (Fig. 2).

Tab. 2. Analysis of variance on protoplast viability (%; n = 3) in *Lilium ledeborii*

Source of variation	Df	MS	F	p value
Cellulase	2	124.16	10.551	0.000
Pectinase	1	6.950	0.591	0.447
Time	1	485.039	41.218	0.000
Cellulase×Pectinase	2	22.198	1.886	0.166
Cellulase×time	2	23.930	2.034	0.146
Pectinase×time	1	0.448	0.038	0.846
Cellulase×Pectinase×time	2	60.561	5.146	0.011
Error	36	11.768		

As a general rule, as the hydrolytic enzyme concentration is increased, more active sites are available for the formation of enzyme-substrate complex (Rastogi, 2003). Hence, the number of viable protoplasts isolated tends to increase correspondingly. Accordingly, an increasing enzyme concentration contributes to an increase in the penetration of enzymes through multilayers of, for example, tightly packed callus cells (Rao and Prakash, 1995). Nonetheless, the cellulose and pectin layers of callus tissue cells may become saturated with enzymes if levels of cellulase and pectinase enzymes are in the order of 2.0% (Kremer and Wood, 1992). Thereafter, addition of more enzymes per unit volume is unable to further increase the numbers of viable protoplasts. Moreover, higher concentrations of enzymes may negatively influence the viability of protoplasts. This reduction in the yield is probably due to over-digestion of the protoplasts by pectinase and cellulase enzymes (Raiker *et al.*, 2008).

The highest yield and viability was obtained from *Anabias nana* when protoplasts were digested from *in vitro* six-week-old leaves with 2% cellulase Onozuka R10, 0.2% pectolyase Y-23, 0.6 M mannitol, 2/5 mM CaCl₂·2H₂O and 5 mM MES [pH 5.6 for 4 h in the dark (Pongchawee *et al.*, 2006)]. Zhang *et al.* (2011) reported the highest yield of more than 1.5×10⁷ protoplasts g⁻¹fresh weight with more than 90% viability was consistently obtained from *Carica papaya* L. by optimised isolation conditions: enzyme combinations of 1.2% cellulase R10, 0.3% macerozyme R10 and 0.44 M d-mannitol, pH=5.8 and incubation for 13h at 26°C in darkness (Zhang *et al.*, 2011).

Ling *et al.* (2010) reported the highest amount of viable protoplasts (1.75±0.68 × 10⁴ protoplasts/gr FW) was obtained from callus of *Eurycoma longifolia* when the sorbitol concentration was maintained at 0.5 M. The optimum enzyme concentration was found to be 1.5% (w/v) of cellulase and pectinase in which 2.75±1.04 × 10⁴ protoplasts/gr FW were isolated. Meanwhile, an incubation period of 3 h with enzyme solution resulted in the maximum yield of protoplasts (5.58±1.46 × 10⁴ protoplasts/gr FW). It's supposed that the observed difference between mentioned results can be because of species and kind of second used enzyme like pectolyase Y-23, which pectinase is used in our experiment instead.

Similarly, a high contact of isolated protoplasts to the centrifuge tubes walls in an increased time term of enzyme

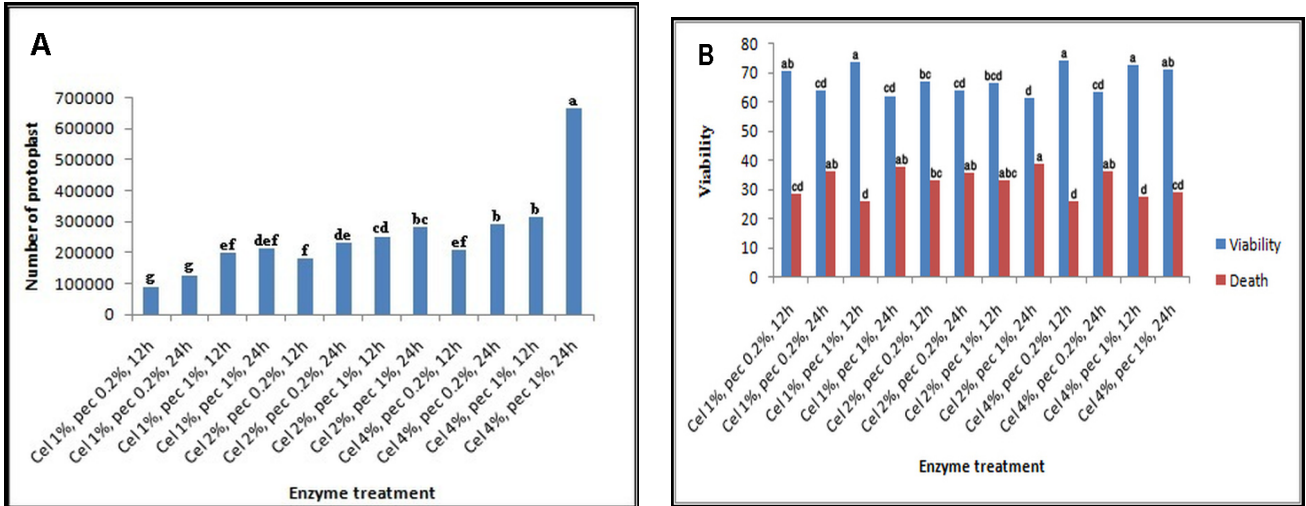


Fig. 1. (A) The mean numbers (n = 3) of isolated protoplasts from *Lilium ledeouborri* leaf mesophyll tissue treated for 12 or 24 h with various different enzyme (Cel = cellulase; Pec = pectinase) cocktail concentrations of cell wall hydrolytic enzymes. Different letters indicate a significant difference at the $p \leq 0.05$ level. (B) The mean viability (%; n = 3) of protoplasts isolated from *Lilium ledeouborri* leaf mesophyll tissue treated for 12 or 24 h with various different enzyme (Cel = cellulase; Pec = pectinase) cocktail concentrations of cell wall hydrolytic enzymes. Different letters indicate a significant difference at the $p \leq 0.05$ level.

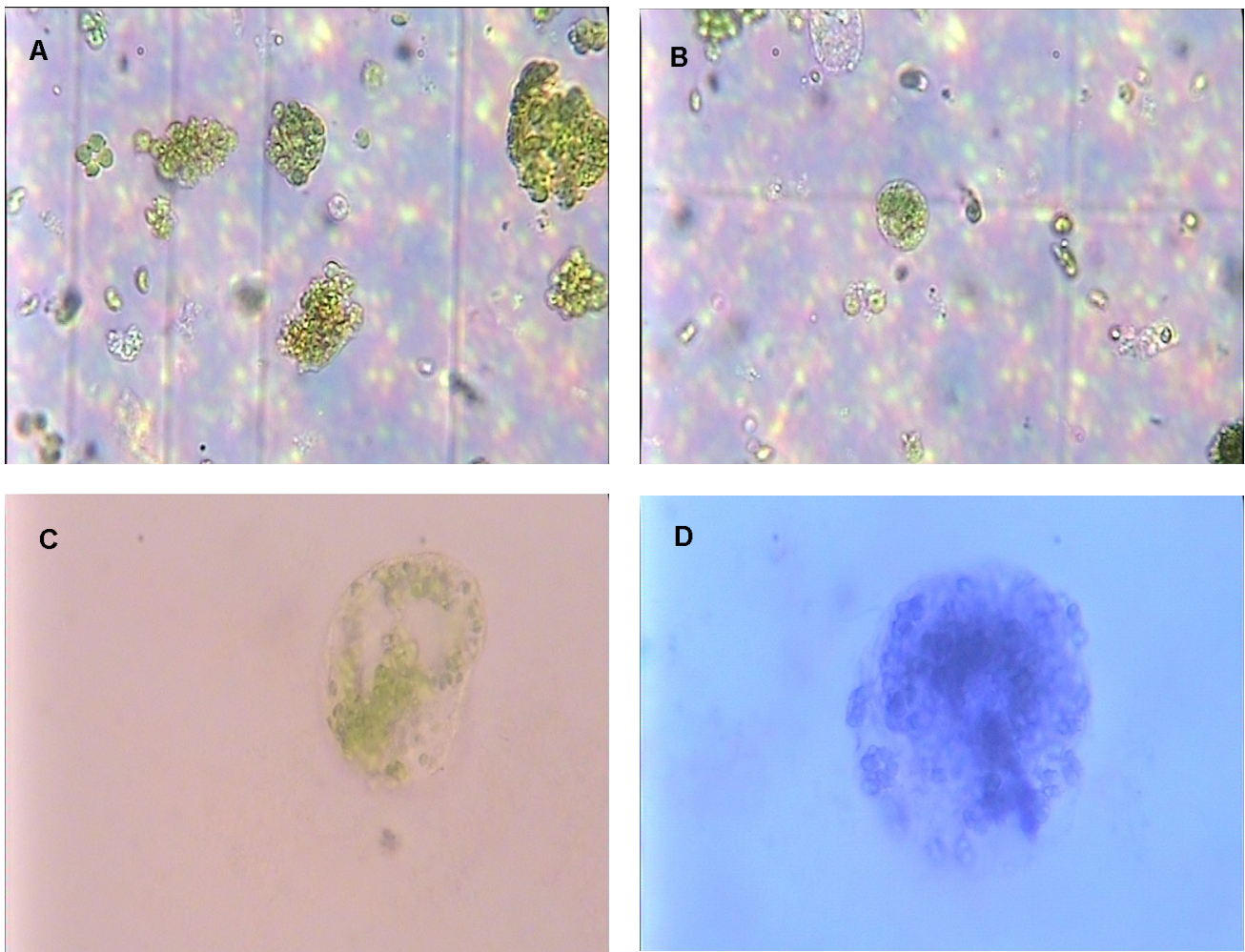


Fig. 2. Protoplasts produced by cell wall hydrolytic enzyme treatments of *Lilium ledouborri* mesophyll tissue. (A) 4% cellulase and 1% pectinase for 24 h. (B) 1% cellulase and 0.2% pectinase for 12 h. (C) viable protoplast. (D) dead protoplast coloured by trypan blue.

treatment had eventually reduced the number of viable protoplasts in the protoplast isolation of *Crocus sativus* L. (Darvishi et al., 2006). In contrast, a digestion period of up to 20 h resulted in the best yield of protoplasts (9.45×10^5 protoplasts/g FW) from the callus tissue of the nitrogen fixing woody plant, *Robinia pseudoacacia* (Kanwar et al., 2009). It's concluded that, the best treatment for isolation of *Lilium* protoplast was 4% cellulase and 1% pectinase for 24 h.

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