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Efficient Protocol for Protoplast Isolation and Plant Regeneration of *Fritillaria imperialis* L.

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ABSTRACT

The present study reports an efficient protocol for isolation and regeneration of protoplasts from callus of *Fritillaria imperialis* L. There is no published method recommended for protoplast isolation and regeneration from *Fritillaria imperialis* L. A range of factors, which influence the success of isolation and regeneration of *F. imperialis* protoplasts, were investigated. From the results obtained, callus Fresh Weight (FW) of 0.4 g produced the highest number of viable protoplasts, which was 1.12×10^5 protoplasts g^{-1} FW. The highest amount of viable protoplasts (1.01×10^5 protoplasts g^{-1} FW) was obtained when the mannitol concentration was maintained at 9% (w/v). The best treatment for isolation of *F. imperialis* protoplast (1.37×10^5 protoplasts g^{-1} FW) was treatment with 2% cellulase and 0.1% pectinase with 9% mannitol for 8 h. For enhancement of the protoplasts division and the percentage of colony formation, different concentrations from Casein Hydrolysate (CH), 2,4-Dichlorophenoxyacetic acid (2,4-D) and Benzyl-Adenine (BA) were used. The results revealed that cell wall and colony formation were better in liquid medium than those on semi-solid medium. The highest plating efficiency (1.26×10^6 per g FW) and highest callus formation were obtained using the medium containing 0.5 mg L^{-1} 2,4-D, 1 mg L^{-1} BA, and 200 mg L^{-1} CH. Micro-calli were formed after one month of culture. Many plantlets were formed on the calli after transfer of the proliferated calli to regeneration medium. The highest plantlet regeneration (100%) was obtained using the medium containing 0.5 mg L^{-1} (Naphthalene Acetic Acid) NAA and 1.5 mg L^{-1} BA.

Keywords: Callus formation, Medium, Protoplast culture, Viability.

INTRODUCTION

Crown imperial or ''Tears of Mary'' (*Fritillaria imperialis* L.) is a perennial plant with high medicinal and ornamental importance. *Fritillaria* genus includes approximately 100 species, 14 important species of which are native to Iran (De Hertogh and LeNard, 1993). In Iran, wild populations of important species, like *F. imperialis* and *F. persica*, are at the risk of rapid eradication because of irregular grazing of *Fritillaria* stands, lack of protecting rules, conversion of the rangelands to dry farmlands, and pest overflow (Ebrahimie *et al*., 2006a). Wild populations of *F. imperialis* are mostly found in high altitudes (> 2,000 m) of western parts of Iran, particularly in two provinces, namely, Chahar Mahal-va-Bakhtiari and Kohkyluyeh-va-Bouyrahmad. The first species of the genus *Fritillaria* were described in 1753, as *F. imperialis* L., *F. persica* L., *F. pyrenaica* L., and *F. meleagris* L. (Linnaeus, 1753). *Fritillaria* is represented worldwide by 7 subgenera, 2 sections, and 165 taxa (Rix, 2001). *Fritillaria imperialis* L. is considered an important source of

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pharmaceuticals. It is one of the native Iranian medicinal plants, and was also very popular for its supposed magical properties. Hence, biotechnology strategies, particularly the somatic hybridization, could provide a promising alternative. The development of protoplast systems has increased the flexibility of plants in biochemical and genetic research (Rao and Prakash, 1995) as well as providing a great prospect in genetic improvement of medicinal plants (Azad *et al*., 2006). The development of protoplast technology and regeneration procedures plays an increasingly important role in the plant improvement through somatic hybridization and protoplast transformation (Umate *et al*., 2005). However, a step towards the plant genetic manipulation and integrated approach of breeding programs is primarily laid on an efficient protocol in protoplast isolation, culture, and regeneration (Duquenne *et al*., 2007). Cells derived from *L. longiflorum* protoplasts subsequently underwent sustained division and gave rise to visible colonies within 3 weeks. Shoots development was induced in the colonies by transferring them to MS-differentiation medium containing NAA and BA at 4 mg L^{-1} and KIN at 2.56 mg L^{-1}, respectively. Protoplasts have been isolated from various genotypes of *Petunia hybrid* (Izhar and Power, 1977), as well as from *P. inflata, P. violocea,* and *P.axillaris* (Dulieu *et al*., 1983). On the other hand, Arnalte *et al.* (1991) reported the procedure for enzymatic isolation of protoplasts from *Digitalis obscura,* which was developed from pollen of this medicinal plant as a tool of genetic improvement of the species. There are no published reports on the isolation, culturing, and regeneration of protoplasts from the *F. imperialis* L. Therefore, the objective of this study was to find out a proper protocol for isolation and culturing of protoplasts from *F. imperialis* L. and regeneration of plantlets from such protoplasts.

MATERIALS AND METHODS

Experimental Designs, Data Collection, and Analysis

In this study, 4 separate experiments were done and each experiment was repeated twice in time. In the first experiment, in order to optimize conditions for protoplasts isolation from *F. imperialis* callus, the effect of fresh weight of callus, osmotic condition, enzymes concentrations, and incubation time were evaluated. In the second experiment, in order to optimize the medium for protoplast growth and cell proliferation, the effect of various plant growth regulator combinations in MS medium [0, 100, 150, 200 and 250 mg L^{-1} Casein Hydrolysate (CH), 0, 0.5,1 and 1.5 mg L^{-1} 2,4-D, 0.2 and 0,0.5,1 and 1.5 mg L^{-1} BA] were tested as a suspension culture based on completely randomized design with factorial arrangement and three replications. In the third experiment of cells proliferated in suspension culture were sub-cultured on semi-solidified MS medium supplemented with various combinations of 2,4-D (0, 0.5, 1, 1.5 mg L^{-1} and BA (0, 0.5, 1, 1.5 mg L^{-1}) and CH (0, 100, 150, 200 and 250 mg L^{-1}) , to determine the growth possibility of protoplast-derived cells on the semi-solid medium. After callus formation, callus colonies were counted. In the fourth experiment, 26 days after callus proliferation, the developed calli in suspension culture were transferred to regeneration medium consisting of semi-solidified MS medium supplemented with NAA (0, 0.5,1 and 0.5 mg L^{-1}) and BA (0, 0.5, 1 and 1.5 mg L^{-1}) based on completely randomized design with factorial arrangement with three replications. The cultures were kept in light conditions of l6 hour light/8 hour dark at 25°C. Cell density was estimated with a Nageotte hematocytometer. Results were expressed as yield per g FW for leaves or calli. The number of callus colonies was evaluated by naked eye. Data analyses were performed using SPSS (SPSS Inc. Version 19.0)

software and MSTATC. Mean comparisons were done using Duncan's Multiple Range Test (DMRT) at a probability level of 0.01.

Protoplast Isolation

Leaves of *in vitro* grown *F. imperialis* L. were used as explants for callus induction. The leaves were isolated and cut into slices of approximately 0.5 cm and were then put on MS basal medium supplemented with 0.8% (w/v) agar and 3% (w/v) sucrose (with pH 5.8). For callus induction, auxins and cytokinins were added to the basal medium in different combinations and their effect on callus induction was studied. The following growth regulators were used in the given concentration: 2.7 μM NAA, 4.4 μM BA, 5 μM 2,4-D and 0.46 μM KIN. Leaf material was incubated at 25°C in the dark and callus formation was scored 6 weeks later. After evaluation, callus was put on fresh medium with the same composition as for the callus induction. The protoplasts were isolated from the 2-week-old callus cultures of *Fritillaria imperialis* maintained on full strength MS medium.

For protoplast isolation, the 2-week-old callus were cut into small pieces and digested by different cell wall digesting enzyme solutions: cellulase (1, 1.5, 2 and 3%), pectinase (0.1, 0.2, 0.5, 1%) and mannitol (9, 11, 13%) (w/v) during 4, 8 and 12 hours. The enzymes were dissolved in Cell Protoplast Washing (CPW) salt solution containing 9% (w/v) mannitol. The pH of the enzyme solutions was adjusted to 5.8. Enzyme solutions were filter-sterilized through 0.2 μm membrane filters (Milipore High-Flow, Sartorius, Germany). The dishes containing callus and enzymes were sealed with Parafilm™ and incubated at 70 rpm for 4, 8 and 12 hours on a rotary shaker in the darkness at 25±2°C. For purification, digested callus 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 (300 rpm for 10 minutes). The pellet was re-suspended in washing solution the same as with enzyme solution but without the enzymes and then centrifuge twice (300 rpm for 10 minutes). Flotation purification was carried out with 21% sucrose at ×100 rpm for 5 minutes. Yields of protoplasts were determined using a double-chamber hemocytometer (Modified-Fuchs Rosenthal rulings, model BS 74B; Weber Scientific International Ltd., Teddington, UK).

Determination of Viable Protoplast:

The viability of purified protoplasts was assessed with uptake and cleavage of trypan blue (material for staining protoplasts, which is used for detection of live from dead protoplast) 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. Optimization of protoplast isolation conditions the conventional "one-factor-at-a-time" method (Fray and Wang, 2006) was employed to optimize the fresh weight of callus, osmotic condition, enzymes concentrations, and incubation time for the protoplasts isolation from *F. imperialis* callus. Only a single factor was changed at a time while other factors were kept constant. All procedures for protoplasts isolation and purification were based on the standard method described earlier, unless otherwise stated.

Fresh Weight of Callus

The effects of FW of *F. imperialis* callus on protoplast yield were tested from the range from 0.2 to 0.4 g. The FW of callus which gave the highest number of viable protoplasts per gram of FW (protoplasts g^{-1} FW) was chosen and used in subsequent experiments (because the released protoplast

at 0.4 g was very high. Moreover, callus explants were not as much as 0.5-0.6 g).

Concentration of Mannitol:

The protoplast suspension was purified in washing medium with different concentrations of mannitol. The effect of mannitol concentrations on the numbers of viable protoplasts isolated was tested at 9, 11 and 13% (w/v).

Concentration of Digestive Enzymes:

The effect of different concentrations of cell wall degrading enzymes on the number of viable protoplasts isolated was also studied. The combination of 1, 1.5, 2, and 3% (w/v) cellulase and 0.1, 0.2, 0.5, and 1% pectinase were added to the protoplasts isolation solution, respectively.

Incubation Time

In this study, the length of incubation period on the number of viable protoplasts isolated was evaluated. The callus tissues were incubated for 4, 8 and 12 hours, respectively, to determine the optimum time required for complete release of protoplasts.

Culture of Protoplasts

Protoplasts were cultured at a density of 1×10^5 protoplasts mL^{-1} and were suspended in 4 mL of liquid media (MS liquid medium with 9% mannitol), in small Petri dishes (5.5 cm diameter). The cells were transferred to Erlenmeyer flasks containing MS liquid medium and incubated at 120 rpm on a rotary shaker in the darkness at 25±2°C five days after protoplast culture. Five mL of fresh medium was added to the culture medium every ten days. Star shaped microcalli developed within 15 days of culture. After the development of microcalli visible by naked eye, the cultures were transferred to the light. The plating efficiency was defined and measured as the ratio of cell number undergoing division to the total cultured protoplast number. Calli were transferred to the semi-solid MS medium at 23°C under fluorescent light (40 $\mu mol\ m^{-2}\ s^{-1}$) in a 16 hour light/8 hour dark condition after one month when calli reaches sizes of 0.5–1.0 mm in diameter.

RESULTS

Effects of Fresh Weight of Callus

The yield of protoplasts was closely dependent on the FW of callus used in the protoplast isolation. The data revealed that the minimum number of viable protoplasts (4.69×10^4 viability) was obtained when 0.2 g of callus was used (Figure 1). Meanwhile, the number of viable protoplasts isolated increased apparently by 47.73 to 80.34% or 1.12×10^5 protoplasts g^{-1} FW when the FW of callus was increased to 0.4 g.

Effects of Concentration of Mannitol

The number of viable protoplasts isolated was strongly affected by the concentration of mannitol used in protoplasts purification process when the FW of callus was fixed at 0.4 g. Meanwhile, a significant improvement of 72.67% in the isolation of viable protoplasts was monitored when 9% (w/v) of mannitol was used in the washing medium (Figure 2) compared to other concentrations. Nonetheless, the use of 11% (w/v) mannitol led to a decrease of 56.1% in the yield of the viable protoplasts. Moreover, the number of viable protoplasts dropped abruptly by 41.69% protoplasts g^{-1} FW when 13% (w/v) mannitol was applied.

Effects of Enzymes Concentration

The results revealed that the number of viable protoplasts obtained was closely

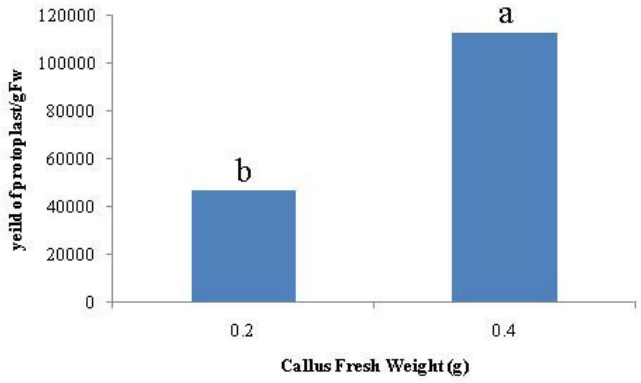

Figure 1. Effects of fresh weight of *F. imperialis* callus on the number of protoplasts isolated. Values followed by the same letter are not significantly different by Duncan's Multiple Range Test (DMRT) multiple comparison test at 0.05 probability level.

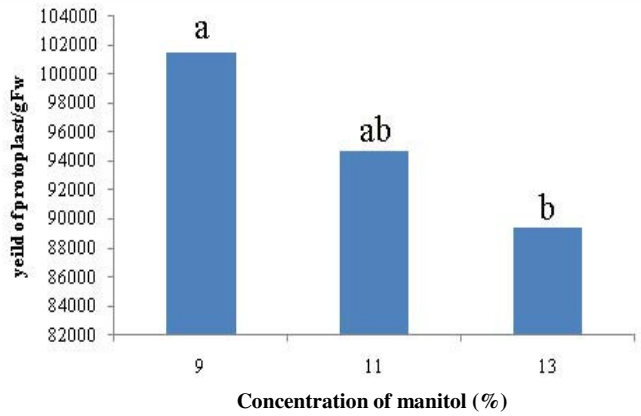

Figure 2. Effects of concentration of mannitol (w/v) in washing medium on the number of viable protoplasts isolated. Values followed by the same letter are not significantly different by Duncan's Multiple Range Test (DMRT) multiple comparison test at 0.05 probability level (The fresh weight of callus was fixed at 0.4 g).

related to the concentration of enzyme used. Analysis of variance showed significant differences between different levels of cellulase, pectinase, and treatment times. Among pectinase treatments, 1% produced the highest number of protoplasts. In case of treatment time, the highest number of protoplasts was for callus treated for 8 hours. Results revealed significant interaction effects of cellulase×time, pectinase×time and on protoplast number. Thus, the best treatment for isolation of *F. imperialis* protoplasts was 2% cellulase and 0.1% pectinase with 9% mannitol for 8 hours. Also, analysis showed significant differences between different levels of cellulase, pectinase and treatment times (Table 1). When the callus tissue was incubated in protoplast isolation solution containing 2% of cellulose and 0.1% pectinase, produced the highest number of protoplasts (1.37×10^5 protoplasts g^{-1} FW). Analysis revealed that the highest and lowest protoplast numbers were produced in media containing 2% cellulase and 0.1% pectinase for 8 hours (1.37×10^5 protoplasts g^{-1} FW) and 3% cellulase and 0.2% pectinase for 12 hours (1.93×10^3 protoplasts g^{-1} FW), respectively. Using 2% cellulase produced the highest viability of protoplasts, with average of 54.38%. Among pectinase treatments, 0.1% produced the highest

Table 1. Analysis of variance of numbers of isolated protoplasts (protoplast mL^{-1}) and protoplast viability *from F. imperialis*.

Source of variation	*df*	MS [a]	
		Protoplasts numbers	Protoplast viability
Cellulase	3	4.061×10^{11} **	3476.065**
Pectinase	3	9.328×10^{10} **	15048.653**
Time	2	3.917×10^{12} **	6129.864**
Cellulase×Pectinase	9	1.42×10^{10} ns	47.039 ns
Cellulase×time	6	4.032×10^{11} **	23.315 ns
Pectinase×time	6	9.09×10^{10} **	123.529**
Cellulase×Pectinase×time	18	1.42×10^{10} ns	61.94**
Error	384	1.642×10^{10}	3.552×10^{8}
CV%		1.28	0.11

[a] ns, * and **: Non significant and significant at probability levels of 5 and 1%, respectively.

viability of protoplasts (60.148%). In case of treatment time, the highest viability of protoplasts was for callus treated for 8 hours (Table 2). This study was also directly related with enzyme-substrate relationship. The effect of enzyme concentrations on the yield of *F. imperialis* protoplast is demonstrated in Figure 3. In this study, the sole variable was the concentration of cellulase and pectinase enzymes and the callus tissue became the limiting factor (Figure 3).

Effects of Incubation Time

The effects of the incubation time on the number of protoplast isolated were examined when 0.4 g of callus, 9% (w/v) of mannitol were used. Figure 4 revealed that the protoplasts yielded in the tissues treated with hydrolytic enzymes increased with the duration of digestion periods, but declined with the extended digestion time. In 4h incubation time, only 1.00×10^{4} protoplasts g^{-1} FW was isolated (Table 2). The optimum incubation period for high viable protoplast yield from 2-week-old *F. imperialis* callus was 8 hours, which yielded 2.91×10^{5} protoplasts g^{-1} FW (Table 2). However, a significant reduction in protoplasts yield was observed when the incubation period was increased to 12 hours (Table 2). The number of viable protoplasts decreased to 1.93×10^{3} protoplasts g^{-1} FW (Table 2). When exposure time was 4 hours, the yield

Table 2. Comparison of different combinations of enzyme treatments on the number of protoplast and viability traits *in F. imperialis.* Mean comparision was done separately for each trait.[a]

Treatment	Protoplast number (g Fw)	Viability (%)
Cellulase 1%	1.26×10^{5} a	47.20 b
Cellulase 1.5%	1.30×10^{5} a	48.57 b
Cellulase 2%	1.37×10^{5} a	54.38 a
Cellulase 3%	9.47×10^{3} b	40.56 c
Pectinase 0.1%	1.37×10^{5} a	60.148 a
Pectinase 0.2%	1.12×10^{5} ab	53.74 b
Pectinase 0.5%	7.48×10^{4} c	43.64 c
Pectinase 1%	7.99×10^{4} bc	33.20 d
time 4 h	1.00×10^{4} b	47.06 b
time 8 h	2.91×10^{5} a	54.49 a
time 12 h	1.93×10^{3} b	41.49 c

[a] Means followed by different letters in each column are significantly different at $P\leq 0.05$.

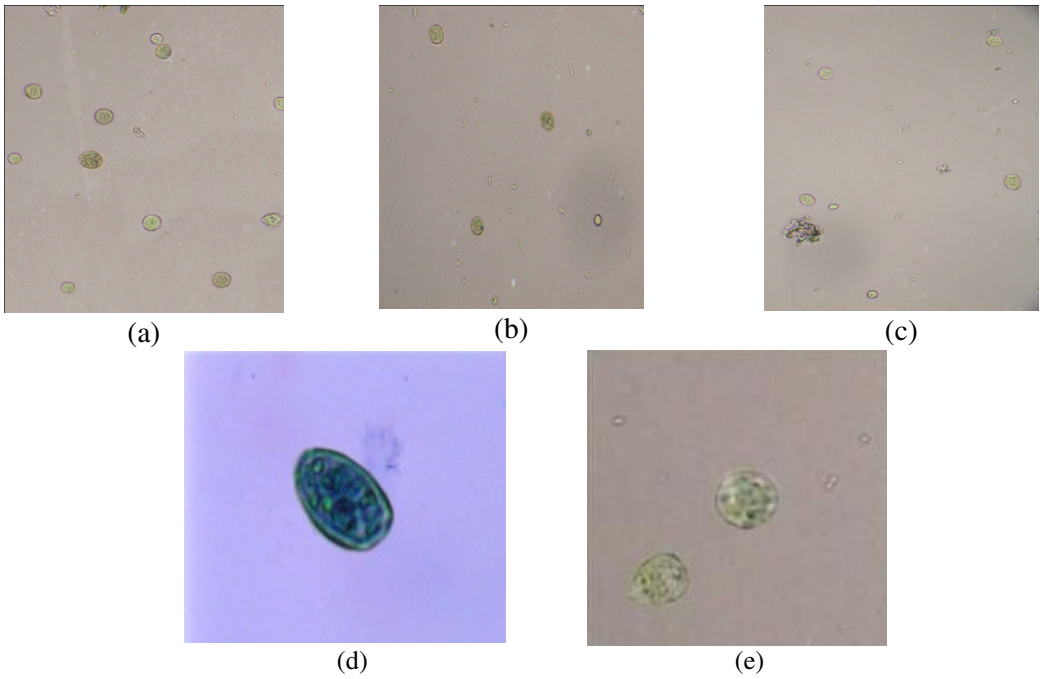

Figure 3. Protoplasts produced by cell wall hydrolytic enzyme treatments of *F. imperialis* callus tissue: (a) 2% Cellulase and 0.1% pectinase for 8 hours; (b, c) 3% Cellulase and 0.2% pectinase for 12 hours; (d) Dead protoplast colored by trypan blue, and (e) Viable protoplast.

dropped apparently to 1.00×10^4 protoplasts g^{-1} FW in 4 hours digestion period (Table 2, Figure 4).

Effect of Different Hormones on Cell Growth and Deviation

The results showed that different concentrations of 2,4-D and BA significantly affected proliferation of protoplast derived cells. Significant interaction effects of 2,4-D×BA, casein hydrolysate×BA, casein hydrolysate×2,4-D and casein hydrolysate×2,4-D×BA were found on cell proliferation (Table 3, Figure 5).

Analysis showed that the highest and lowest cell proliferation were produced in MS suspension medium containing 0.5 mg L^{-1} 2,4-D, 1 mg L^{-1} BA and 200 mg L^{-1} CH (1.26×10^6 cell g^{-1} FW), and MS media without PGR (8.2×10^5 cell g^{-1} FW), respectively. However, other MS suspension media containing 0.5 mg L^{-1} 2,4-D , 1.5 mg L^{-1} BA and 200 mg L^{-1} casein hydrolysate or 1 mg L^{-1} 2,4-D , 1.5 mg L^{-1} BA and 150 mg L^{-1} casein hydrolysate or 0.5 mg L^{-1} 2,4-D , 1 mg L^{-1} BA and 150 mg L^{-1}casein hydrolysate and as well as 0.5 mg L^{-1} 2,4-D, 1 mg L^{-1} BA and 100 mg L^{-1}casein hydrolysate produced significantly highest density of cells. Hence, the latest mentioned media was not used in the next experiments. Thus, the best treatment for proliferation and growth of *F. imperialis* cells was MS medium supplemented with 0.5 mg L^{-1} 2,4-D, 1 mg L^{-1} BA and 200 mg L^{-1} CH (Table 4). The first cell divisions were observed 48 hours after protoplast culture. Cell density was measured every 5 days and the first density measurement was done 15 days after protoplast culture (Figure 5).

Callus Mass Formation from Plating of Cell Suspension on Solid MS Medium

The results showed that growth of plated cells and formation of calli (detectable by

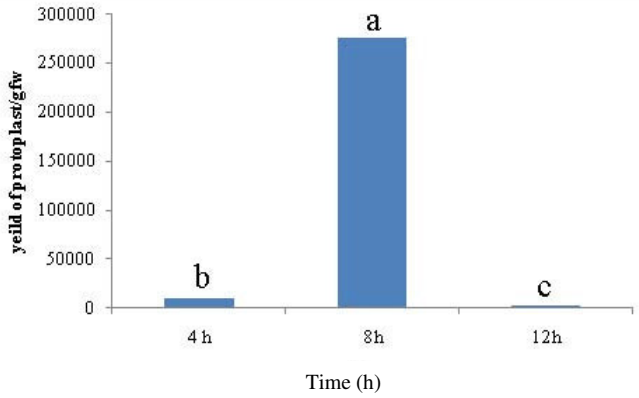

Figure 4. Effects of different incubation periods on the number of isolated viable protoplasts. Values followed by the same letter are not significantly different by Duncan's Multiple Range Test (DMRT) multiple comparison test at 0.05 probability level (The fresh weight of callus was fixed at 0.4 g).

Table3. Analysis of variance of effects of casein hydrolysate and different plant growth regulators on the proliferation and growth protoplasts of *F. imperialis.*

Source of variation	*df*	MS[a]
BA	3	1.307×10^{11} **
2,4-D	3	2.118×10^{11} **
Casein hydrolysate	4	6.380×10^{10} **
2,4-D×BA	9	5.078×10^{10} **
Casein hydrolysate×BA	12	6.998×10^{9} **
Casein hydrolysate×2,4-D	12	1.909×10^{10} **
Casein hydrolysate×2,4-D×BA	36	7.630×10^{9}**
Error	160	6.612×10^{8}
CV%		2.44

[a] ns, * and **: Non significant and significant at probability level of 5 and 1%, respectively.

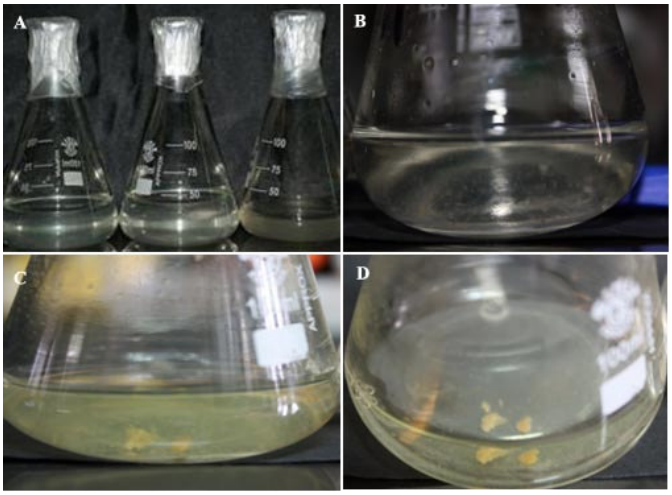

Figure 5. Developmental stages of protoplast in culture suspension: (A) culture suspension contains released protoplast; (B) Cell proliferation and growth after two days and turbid suspension medium, and (C, D) Formation of cell masses after 14 days and cell mass enlargement and callus formation after 20 days of culture, respectively.

Table 4. The mean effect of different combinations of hormones on density of cells in *F. imperialis.*

2,4-D (mg L^{-1})	BA (mg L^{-1})	Casein hydrolysate (mg L^{-1})	Dencity of cells (in 1 mL)[a]
0	0	0	8.2×10^{5}g
0.5	0.5	200	1.05×10^{6} e
0.5	0.5	250	1.04×10^{6}f
0.5	1	150	1.05×10^{6}d
0.5	1	200	1.26×10^{6} a
0.5	1.5	200	1.18×10^{6}b
1	1.5	150	1.12×10^{6}c

[a] Means followed by different letters in each column are significantly different at $P\leq 0.05$.

naked eye) on semi-solidified medium were significantly influenced by different combinations of plant hormones and casein hydrolysate (Table 5). Means comparison revealed that the highest and lowest callus induction from plated cell on semi-solidified MS medium were produced on media containing 0.5 mg L^{-1} 2,4-D and 1 mg L^{-1} BA with 200 mg L^{-1} casein hydrolysate (35.33%) and 0 mg L^{-1} 2,4-D and 0 mg L^{-1} BA and 0 mg L^{-1} CH(0), respectively (Table 6, Figure 6).

Plant Regeneration

The results showed that different concentrations of NAA and BA significantly affected plant regeneration of *Fritillaria imperialis* L. Significant interaction effects of NAA×BA were found on regeneration (Table 7).

The results showed that the highest regeneration were produced in MS medium containing 0.5 mg L^{-1} NAA, 1.5 mg L^{-1} BA (100%) (Figure 7). However, other media containing 0.5 mg L^{-1} NAA and 1 mg L^{-1} BA (%66.6), 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} BA (%55.5), 1 mg L^{-1} NAA and 1.5 mg L^{-1} BA (%33.33) and as well as 1 mg L^{-1} NAA, 1 mg L^{-1} BA (%22.2) produced significantly highest regeneration (Figure 7). Thus, the best treatment for growth and regeneration of *F. imperialis* was MS medium supplemented with 0.5 mg L^{-1} NAA and 1.5 mg L^{-1} BA (Figure 7).

Table 5. Analysis of variance of the effects of different treatments on growth efficiency of plated cells of *F. imperialis* on solidified MS medium and formation of callus in *F. imperialis.*

Source of variation	*df*	MS
Treatment	13	353/753**
Error	28	1.405
CV%		0.12

**: Significant at probability level of 1%

DISCUSSION

No reports were found on protoplast culture and regeneration in *Fritillaria imperialis* L. Thus, 0.2 g of friable and yellow embryogenic suspension cell cultures was chosen to be used in the protoplast isolation of *Cinnamomum camphora* L. (Du and Bao, 2005).

In principle, the cellulase and pectinase enzyme could hydrolyze pectin and cellulose layer of the cell wall of *F. imperialis* callus tissues within a limited area before dissociation of the enzyme occurred (Lenting and Warmoeskerken, 2001). Since the enzyme concentration was constant throughout the experiment, an increase in the FW of callus tissue led to more effective collisions between the callus cells and the enzymes per unit time (Royal Society of Chemistry, 2005). Indeed, a further increase in FW of callus exceeded the number of active sites that were available for the enzymes (Kashyap, 2001). The depletion of

Table 6. The effects of different treatments on callus formation from plated cells of *F. imperialis.*

2,4-D (mg L^{-1})	BA (mL^{-1})	Casein hydrolysate (mg L^{-1})	Number of callus mass formed in each petridish[a]
0	0	0	0 [i]
0.5	0.5	100	1.66 [ghi]
0.5	0.5	150	2 [ghi]
0.5	0.5	200	3.33 [gf]
0.5	0.5	250	1 [hi]
0.5	1	100	10.33 [e]
0.5	1	150	17.66 [d]
0.5	1	200	35.33 [a]
0.5	1	250	5 [f]
0.5	1.5	100	3 [fgh]
0.5	1.5	150	4.33 [f]
0.5	1.5	200	24.33 [b]
0.5	1.5	250	1.66 [ghi]
1	1.5	150	20 [c]

[a] Means followed by different letters in each column are significantly different at $P \leq 0.05$.

Table 7. Analysis of variance of different treatments on plant regeneration from cultured protoplasts in *F. imperialis*.

Source of variation	*df*	MS
NAA	3	6565.720**
BA	3	3232.72**
BA×NAA	9	969.632**
Error	32	162.005
CV%		0.59

**: Significant at probability level of 1%.

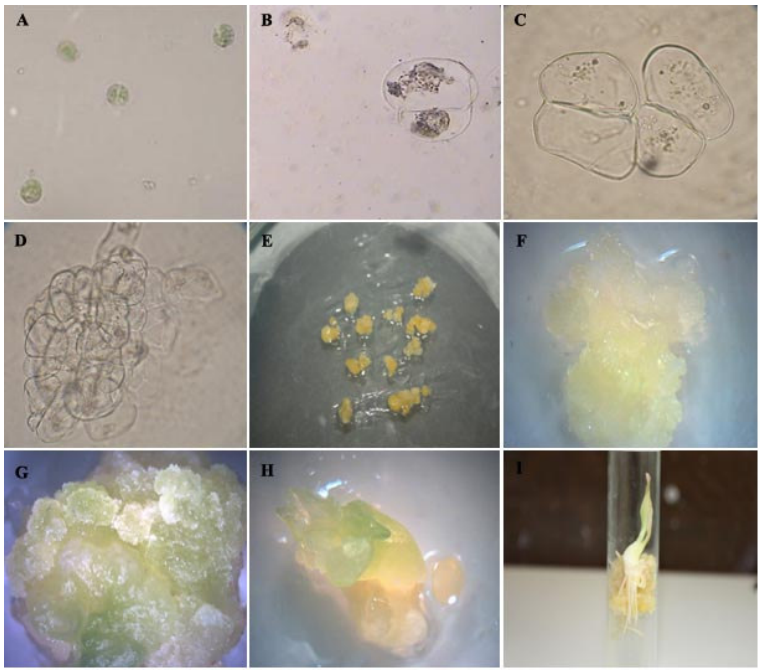

Figure 6. General overview of protoplast culture and regeneration procedure developed for *F. imperialis*: (A) Isolated protoplasts from callus; (B) First division after 48 h of culture; (C) Second division after 4 days of culture; (D, E) Colony formation after 3 weeks of culture, F) Plate of cell suspension and callus formation can be detected with the naked eye after 25 days, (G, H) Callus regenerated, and (I) Regenerated plants from protoplasts.

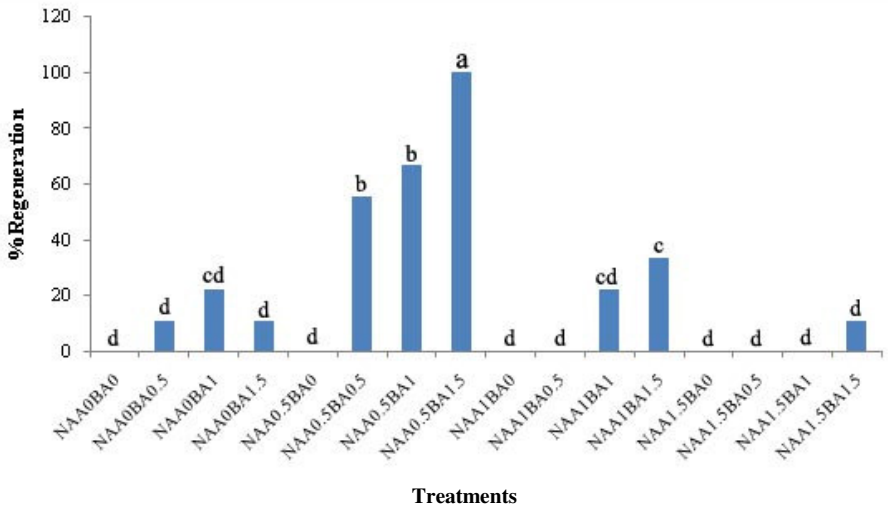

Figure 7. The effect of different treatments on plant regeneration in *F. imperialis.*

the enzymes reduced the effective collision between the active site to their targeted substrate when the number of cells per unit volume increased (Nelsestuen and Martinez, 1999). Generally, the mannitol acted as flotation agent and sole osmotic stabilizer in isolating a viable protoplast effectively (Jullien *et al*., 1998). The freshly isolated protoplasts were prone to breakage when sorbitol solution was not added to the washing medium. Without osmoticum like sorbitol, the water molecules diffused into the protoplasts and caused the cell to rupture (Karp, 2005). The concentration and type of osmotic stabilizer required for successful protoplasts isolation varies with the plant species and growing conditions. For example, Sinha *et al*. (2003) reported that the best yield of protoplasts isolated from *Lupinus albus* L. was optimal at 0.5 (w/v) of mannitol. As the enzyme concentration increased, more active sites were available for effective collisions in the formation of enzyme-substrate complex (Rastogi, 2003). Hence, the number of viable protoplasts isolated was also increased correspondingly. Since the tissues were readily attacked by the enzymes, an increasing enzyme concentration contributed to an increase in the penetration ability of enzymes through multilayer of tightly packed cells in callus (Rao and Prakash, 1995). However, the cellulose and pectin layer of the callus tissues would be saturated with the enzymes in subsequent increases of cellulose and pectinase enzymes to 2.0% (Kremer and Wood, 1992). Therefore, an addition of enzymes per unit volume was unable to further increase the number of viable protoplasts. In contrast, a higher concentration of enzymes negatively influenced the viability of the protoplasts. The reduction in the yield of viable protoplasts in excess enzyme concentration was probably due to over-digestion of the protoplasts by pectinase and cellulose enzymes (Raikar *et al*., 2008). Similarly, a high contact of isolated protoplasts to the centrifuge tubes walls in an increased time term of enzyme 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 hours resulted in the best yield of protoplasts (9.45×10^5 protoplasts g^{-1} FW) from the callus tissue of the nitrogen fixing woody plant, *Robinia pseudoacacia* (Kanwar *et al*., 2009) and the best treatment for isolation of *Lilium* protoplasts was 4% cellulase plus 1% pectinase for the 24 hours treatment time (Chamani *et al*., 2012).

The efficiency of protoplast isolation and growth depends on many factors, such as the enzyme mixture, the presence and type of growth regulator and *in-vitro* culture (Assani *et al*., 2001). A liquid medium was better than an agarose-solidified PCA medium for further growth of isolated protoplasts, although in many crops agarose-solidified media were used. They showed that more frequent browning occurred in an agarose-solidified medium than in a liquid medium. This browning is probably caused by the oxidation of phenolic compounds, which are released from cultured plant cells into the medium (Saxena and Gill, 1986). Oxidation causes severe damage to plant cells or tissue, which consequently becomes arrested in growth. In a liquid medium, this toxic compound might be diluted, thus showing less browning than an agarose-solidified medium does. Ochatt and Power (1988a) reported that casein hydrolysate was needed for sustaining protoplast division of Williams pear as a source of amino acids. In these experiments, we used yeast extract instead of casein hydrolysate. These results are supported with the findings of Ochatt and Power (1988b) who used protoplasts of several woody fruit crops. Therefore, the positive effect of casein hydrolysate or yeast extract and amino acids on cell division proved its successful effect in protoplast cultures.

Result indicated that combination of BA and 2,4-D in high concentration inhibited protoplast division. This result was consistent with earlier findings that the combined optimal auxin and cytokinin were relatively effective for cell division in petal protoplast of *Petunia hybrid* (Oh and Kim, 1994), and in cell suspension protoplast of *Allium cepa* (Karim and Adachi, 1997). In these experiments, protoplasts were cultured either in liquid and solid MS medium comprising 1×10^5 and 1×10^5 protoplasts mL^{-1}. Division of protoplasts obtained in liquid MS medium at optimal density was 1.26×10^6 protoplasts mL^{-1}. The density of protoplasts influenced the initiation of cell divisions, as has been reported in oat by Hahne *et al*. (1990). The suspension-derived protoplasts of vetiver did not divide in gelrite (Kisaka *et al*., 1998). During the present study, cell-wall regeneration, cell division, and callus formation were obtained. Among the plant growth regulators we tested, only the combination of 2,4-D and BA induced cell division. In earlier studies on rose mesophyll protoplasts, NAA and BA were the most efficient growth regulators for the regeneration of microcalli (Marchant *et al*., 1997). In lily protoplasts, the addition of picloram to the culture medium was critical for development of microcalli (Horita *et al*., 2002). The number of microcalli we obtained was close to those obtained in earlier studies in banana (Assani *et al*., 2001). The high concentration of auxin, does not make root formation but makes callus formation (Pierik, 1998). Shoot organogenesis depends on many parameters, including the genotype, protoplast-derived material, plant growth regulators, culture system, and exposition time of protoplasts on nurse cells (Chabane *et al*., 2007). Previous investigations showed the impact of genotype on plant regeneration from protoplasts in apple and banana (Assani *et al*., 2002). Chang (1999) reported that the optimum callus formation from inflorescence explants of *lilium* was obtained in medium containing 3 mg L^{-1} 2,4-D and 0.25 mg L^{-1} BA. In another experiment, Naik and Nayak (2005) reported that callus induction in scale explants of *Ornithogalum virens* was obtained in medium containing 1-4 mg L^{-1} 2,4-D and 2 mg L^{-1} BA. Chen (2005) also stated that the highest percentage of callus induction from another culture of Narcissus was obtained in medium containing 1 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BA. The main plant growth regulators such as auxin and cytokinin, alone or in combination, are generally essential for efficient protoplast division in plant systems (Davey *et al*., 2005). Plant growth regulator concentrations and combinations need to be optimized for each protoplast development step. The following plant growth regulators were tested in our preliminary experiments:

2,4-D, BA, NAA and casein hydrolysate. Only the combination of 2,4-D and BA induced sustained cell divisions and callus formation. None of the plant growth regulators induced plant regeneration, which may be related to the negative interaction between those plant growth regulators and some metabolites produced by callus tissues. Nagata and Takede (1984) succeeded in isolating of protoplasts from *Nicotiana tabacun* L. leaves using enzyme solution. They isolated 10^7 protoplasts from 1 g fresh weight of tobacco leaves. After 3 weeks, shoots were induced in the colonies by transferring them into differentiation medium containing NAA and BA at 4 mg L^{-1} and KIN at 2.56 mg L^{-1}. Concentrations of 0.2 mg L^{-1} 2,4-D, 1 mg L^{-1} NAA and 0.5 mg L^{-1} Zeatin, was produced the highest protoplast regeneration and cell division from L. pyrenacium (Pongchawee *et al*., 2006). They also proved that, addition of Zeatin (1 mM) and NAA (10 mM) gives the normal size of the colonies formed. Changing protoplast culture medium to 5.4 mM NAA and 2.3 mM Zeatin was suitable for protoplast regeneration. Therefore, that was the appropriate density of cells in the medium (Tian *et al*., 1999). Also, culture of protoplasts onto 1/2 strength MS-medium containing 0.01 mg L^{-1} NAA, 0.5 mg L^{-1} BA had a high plant regeneration from *Hypericum perforatum* (Saker *et al*., 1999).

CONCLUSIONS

This study developed a protocol for isolation and plant regeneration from protoplasts of *Fritillaria imperialis* L., which is native to Iran. Our results show the best treatment for isolation of protoplast, growth, cell division, cells suspension culture, callus mass formation from plating of cell suspension on solid MS medium, and plant regeneration. This is, to our knowledge, the first report of plant regeneration from protoplasts of *Fritillaria imperialis* species. We hope the protocol can be applied to the regeneration of protoplasts from other plant species as well.

Abbreviations

BA: 6-BenzylAdenine, CH: Casein Hydrolysate, CPW: Cell Protoplast Washing, FW: Fresh Weight, KIN: Kinitin, MS: Murashige and Skoog nutrition medium, NAA: NaphthAleneacetic Acid, 2,4-D: 2,4-Dichlorophenoxyacetic acid.

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موثرترین روش برای جداسازی و باززایی پروتوپلاست از کالوس گیاه لاله واژگون

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چکیده

مطالعه حاضر موثرترین روش برای جداسازی و باززایی پروتوپلاست از کالوس گیاه لاله واژگون را گزارش می کند. این روش توصیه شده برای جداسازی و باززایی گیاه لاله واژگون از طریق کشت پروتوپلاست قبلا چاپ نشده است. فاکتورهای مختلفی که موفقیت در جداسازی و باززایی پروتوپلاست گیاه لاله واژگون را تحت تاثیر قرا می دهد بررسی شدند. نتایج بدست آمده نشان دادند که، ۰/۴ گرم وزن کالوس با میانگین $۱/۱۲\times۱۰^{۵}$ پروتوپلاست در یک میلی‌لیتر بیشترین تعداد پروتوپلاست زنده را داشت. بیشترین مقدار پروتوپلاست های زنده با میانگین $۱/۰۱\times۱۰^{۵}$ هنگامی که غلظت مانیتول را در ۹٪ نگه داشتیم بدست آمد. بهترین تیمار برای جداسازی پروتوپلاست از گیاه لاله واژگون (با میانگین $۷/۸\times۱۰^{۵}$) ، تیمار آنزیمی سلولاز ۲ درصد، پکتیناز ۰/۱ درصد با مانیتول ۹٪ و زمان ۸ ساعت بود. برای بدست اوردن تقسیم پروتوپلاست و درصد کلونی های تشکیل یافته غلظت های مختلفی از کازئین هیدرولیزات، توفور دی، و بنزیل آدنین استفاده شد. نتایج نشان داد که تشکیل دیواره سلولی و کلونی در محیط مایع نسبت به محیط نیمه جامد آگارز بهتر بود. بیشترین تراکم کشت و تشکیل کالوس در محیط کشت حاوی ۰/۵ میلی‌گرم در لیتر 2,4-D ، ۱ میلی‌گرم در لیتر BA به همراه ۲۰۰ میلی‌گرم در لیتر کازئین هیدرولیزات با میانگین $۱/۲۶\times۱۰^{۶}$ بدست آمد. کالوس‌های کوچک بعد از یک ماه کشت تشکیل شدند. گیاهچه‌های زیادی پس از انتقال کالوس‌های رشد یافته به محیط کشت حاوی تنظیم کننده‌های رشد گیاهی تشکیل شدند. محیط کشت MS حاوی ۰/۵ میلی‌گرم در لیتر NAA به همراه با ۱/۵ میلی‌گرم در لیتر BA بیشترین باززایی با میانگین ۱۰۰ درصد تولید شد.