Scientific Papers. Series B, Horticulture. Vol. LVIII, 2014
Print ISSN 2285-5653, CD-ROM ISSN 2285-5661, Online ISSN 2286-1580, ISSN-L 2285-5653

HOW TO REGENERATE PLANTLETS FROM PROTOPLASTS OF FRITILLARIA IMPERIALIS

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Abstract

There is no special method recommended for protoplast isolation and regeneration from Fritillaria imperialis L. The present study reports the isolation and regeneration of protoplasts from callus of Fritillaria imperialis L. A range of parameters which influence the 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 FW. The best treatment for isolation of Fritillaria imperialis protoplast (7.8 × 10^5 protoplasts/g FW) was 2% cellulase and 0.1% pectinase with 9% manitol for 8 h. For enhancement of the protoplasts division and the percentage of colony formation, different concentrations from casein hydrolysate, 2,4-D and BA were used. The results revealed that cell wall and colony formation were better in a liquid medium than those on a semi-solid medium. The highest plating efficiency (1.26×10^6 per gr FW) and highest callus formation was obtained by using a medium containing 0.5 mg l^{-1} 2,4-D,1 mg l^1 BA and 200 mg l^{-1} casein hydrolysate. 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 by using a medium containing 0.5 mg l^{-1} NAA, 1.5 mg l^{-1} BA.

Key words: *Callus formation, Fritillaria imperialis L, Plant regeneration, Protoplast culture*

INTRODUCTION

Crown imperial (*Fritillaria imperialis* L.) or ''*Tears of Mary*'' (because of great drops of nectar at the petal base) is a perennial plant with high medicinal and ornamental importance. Approximately, *Fritillaria* genus includes 100 species which 14 important species 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, changing the pastures 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, Chahar Mahal-va-Bakhtiari and Kohkyluyeh-va-Bouyrahmad. The species of the genus *Fritillaria* were first 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). As the production of a better adapted *Fritillaria imperialis* hybrid through conventional plant breeding techniques is difficult and time consuming. Hence, biotechnology strategies particularly the somatic hybridization could provide a promising alternative. The development of protoplast systems has enlarged the flexibility of plants in biochemical and genetic research (Rao and Prakash, 1995) as well as provides a great prospect in genetic improvement of medicinal plants (Azad et al., 2006). The development of protoplast technology and regeneration procedures played an increasingly significant 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 protoplasts subsequently undergo sustained division and gave rise to visible colonies within 3 weeks. Shoots formation was induced in the colonies by transferring them to MS-differentiation medium (Murashige and Skoog,

1962) containing NAA and BA at 4 mg l^{-1} and Kin at 2.56 mg l^{-1}, respectively. Shoots were transferred to White's basal medium to induce root formation. 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,* it 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 *Fritillaria imperialis* L. Therefore, the objective of this study was to find out a proper protocol for isolation and culturing of protoplasts from *Fritillaria imperialis* L. and regeneration of plantlets from such protoplasts. *Fritillaria imperialis* L. is considered an important source of pharmaceuticals. It is one of the native Iran medicinal plants, and was also very popular for its supposed magical properties.

MATERIALS AND METHODS

Culture of protoplasts

Protoplasts were cultured at a density of 1×10^5 protoplasts/ml. protoplasts were suspended in 4 ml of liquid media (MS without agar, with 9% mannitol), in small Petri dishes (5.5 cm diameter). 5 days after protoplast culture, 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. After 10 days, every time, 5 ml of fresh medium was added to the culture medium. 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 defined and measured as the ratio of cell number undergoing division to the total cultured protoplast number. After one month when the calli attained sizes of 0.5–1.0 mm in diameter, they were transferred to the semi-solidified MS medium at 23°C under fluorescent light (40 µmol per m^2/s) in a 16/8 h of day/night regime in the culture cabinets.

Experimental designs, data collection and analysis

In this study three separate experiments were done and each experiment was repeated twice. In first 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 (Cas), 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 second experiment, to determine the growth possibility of protoplast-derived cells on the semi-solid medium, all 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 casein hydrolysate (Cas) (0, 100, 150, 200 and 250 mg l^{-1}). After callus formation, callus mass were counted. In third experiment, after 26 days of 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}), 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 hrs/day at 25°C. Cell density was estimated with a Nageotte hematocytometer. Results were expressed as yield per gr FW for leaves or calli. Callus mass 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 0.05.

RESULTS AND DISCUSSION

Effect of different hormones on cell growth and deviation

The results of ANOVA showed that different concentrations of 2,4-D and BA significantly ($P\leq0.01$) affected proliferation of protoplast derived cells. Significant ($P\leq0.01$) 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.

Means comparison by DMRT 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} casein hydrolysate (1.26×10^6 cell/g FW), and 0 mg l^{-1} 2,4-D and 0 mg/lit BA (8.2×10^5 cell/gr FW), respectively (Tab 1). 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, 1 mg l^{-1} 2,4-D , 1.5 mg l^{-1} BA and 150 mg l^{-1} casein hydrolysate, 0.5 mg l^{-1} 2,4-D , 1 mg/l 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 did not use in 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} casein hydrolysate. 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.

Callus mass formation from plating of cell suspension on solid MS medium

The results of ANOVA showed that growth of plated cells and formation of calli (detectable by naked eye) on semi-solidified medium were significantly ($P\leq0.01$) influenced by different combinations of plant hormones and casein hydrolysate. 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/lit BA and 0 mg l^{-1} casein hydrolysate (0) respectively (Table 2).

Table 1. 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 1ml)
0	0	0	8.2×10^5z
0	0	100	8.42×10^5z
0	0	150	8.49×10^5z
0	0	200	8.59×10^5z
0	0	250	8.50×10^5z
0	0.5	0	9.49×10^5hijklmnop
0	0.5	100	9.53×10^5hijklmnopqr
0	0.5	150	9.41×10^5ijklmnopqrstu
0	0.5	200	9.57×10^5hijklmnop
0	0.5	250	9.66×10^5ghi
0	1	0	9.48×10^5hijklmnopqrst
0	1	100	9.58×10^5hijklmnopqrst
0	1	150	9.48×10^5ijklmnopqrst
0	1	200	9.56×10^5hijklmnopq
0	1	250	9.53×10^5hijklmnopqrst
0	1.5	0	9.36×10^5hijklmnopqrst
0	1.5	100	9.44×10^5hijklmnopqrst
0	1.5	150	9.60×10^5hijklmnopqr
0	1.5	200	9.49×10^5hijklmnopqr
0	1.5	250	9.7×10^5ghij
0.5	0	0	9.2×10^5tuvw
0.5	0	100	9.3×10^5 rstuvw
0.5	0	150	9.55×10^5hijklmnopq
0.5	0	200	9.36×10^5klmnopqrstu
0.5	0	250	9.62×10^5hijklmnopqrs
0.5	0.5	0	9.84×10^5hijk
0.5	0.5	100	1.02×10^6 fg
0.5	0.5	150	1.03×10^6 f
0.5	0.5	200	1.05×10^6 ef
0.5	0.5	250	1.04×10^6f

0.5	1	0	9.81×10^{5} hijkl
0.5	1	100	1.04×10^{6}de
0.5	1	150	1.05×10^{6}d
0.5	1	200	1.26×10^{6} a
0.5	1	250	1.04×10^{6}de
0.5	1.5	0	9.7×10^{5}hijk
0.5	1.5	100	1.05×10^{6}f
0.5	1.5	150	1.00×10^{6}gh
0.5	1.5	200	1.18×10^{6}b
0.5	1.5	250	9.97×10^{5} hijklmno
1	0	0	8.98×10^{5}uvw
1	0	100	9.38×10^{5}nopqrstuv
1	0	150	9.34×10^{5}ijklmnopqrst
1	0	200	9.41×10^{5}ijklmnopqrst
1	0	250	9.47×10^{5} hijklmnopq
1	0.5	0	9.16×10^{5}qrstuvw
1	0.5	100	9.26×10^{5}opqrstuv
1	0.5	150	9.38×10^{5}ijklmnopqrstu
1	0.5	200	9.41×10^{5}jklmnopqrstu
1	0.5	250	9.54×10^{5}hijklmnopqrst
1	1	0	9.16×10^{5} vwx
1	1	100	9.25×10^{5} mnopqrstuv
1	1	150	9.45×10^{5}hijklmnop
1	1	200	9.26×10^{5} nopqrstuv
1	1	250	9.57×10^{5}pqrstuv
1	1.5	0	8.97×10^{5}xyz
1	1.5	100	9.95×10^{5}ghi
1	1.5	150	1.12×10^{6}c
1	1.5	200	9.95×10^{5}ghi
1	1.5	250	9.21×10^{5} stuvw
1.5	0	0	8.93×10^{5}z
1.5	0	100	9.52×10^{5}ijklmnopqrst
1.5	0	150	9.50×10^{5} hijklmnopqrst
1.5	0	200	9.41×10^{5}ijklmnopqrstu
1.5	0	250	9.51×10^{5}hijklmnopqrst
1.5	0.5	0	9.06×10^{5} wxy
1.5	0.5	100	9.57×10^{5}hijklmn
1.5	0.5	150	9.59×10^{5} hijklmnop
1.5	0.5	200	9.64×10^{5}hijklm
1.5	0.5	250	9.33×10^{5}klmnopqrstu
1.5	1	0	8.90×10^{5}xyz
1.5	1	100	9.33×10^{5} klmnopqrstu
1.5	1	150	9.40×10^{5} hijklmnopqrst
1.5	1	200	9.44×10^{5} jklmnopqrstu
1.5	1	250	9.31×10^{5}lmnopqrstuv
1.5	1.5	0	8.87×10^{5}yz
1.5	1.5	100	9.36×10^{5} opqrstuv
1.5	1.5	150	9.35×10^{5} klmnopqrstu
1.5	1.5	200	9.43×10^{5}hijklmnopqrst
1.5	1.5	250	9.38×10^{5}jklmnopqrstu

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

Table 2. The effects of different treatment on callus formation from plated cells of *F. imperialis.*

2,4-D (mg l^{-1})	BA (mg l^{-1})	casein hydrolysate (mg l^{-1})	Number of callus mass formed in each petridish
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}

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

Plant regeneration

The results of ANOVA showed that different concentrations of NAA and BA significantly ($P \leq 0.01$) affected plant regeneration of *Fritillaria imperialis* L. Significant ($P \leq 0.01$) interaction effects of NAA× BA were found on regeneration.

Means comparison by DMRT showed that the highest and lowest regeneration were produced in MS medium containing 0.5 mg l^{-1} NAA, 1.5 mg l^{-1} BA (%100), and 0 mg l^{-1} NAA and 0 mg/lit BA (0), respectively (Fig. 1**)**. However, other media containing 0.5 mg l^{-1} NAA and 1 mg l^{-1} BA (%66.66), 0.5 mg l^{-1} NAA and 0.5 mg l^{-1} BA (%55.55), 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 (%22.22) produced significantly highest regeneration. (Figure1). 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 1).

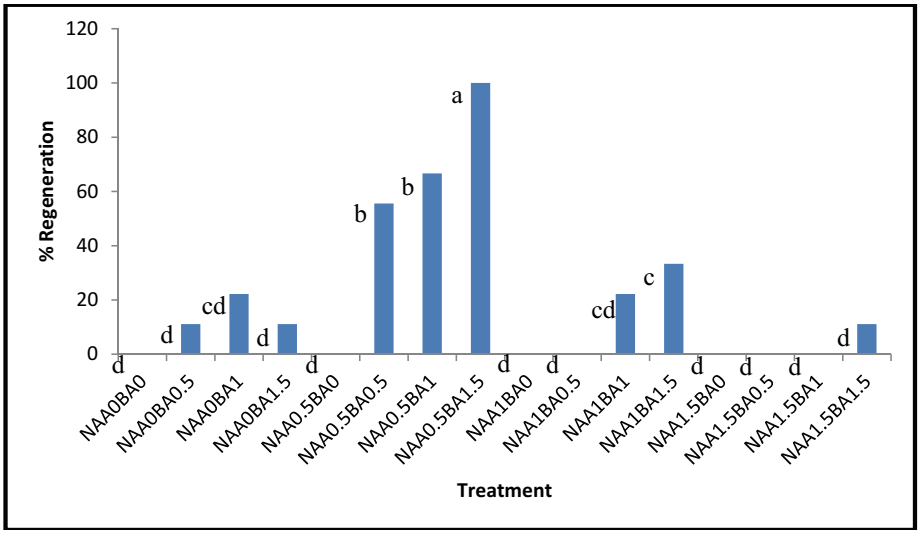

Figure 1. The effect of different treatments on plant regeneration in *F. imperialis.*

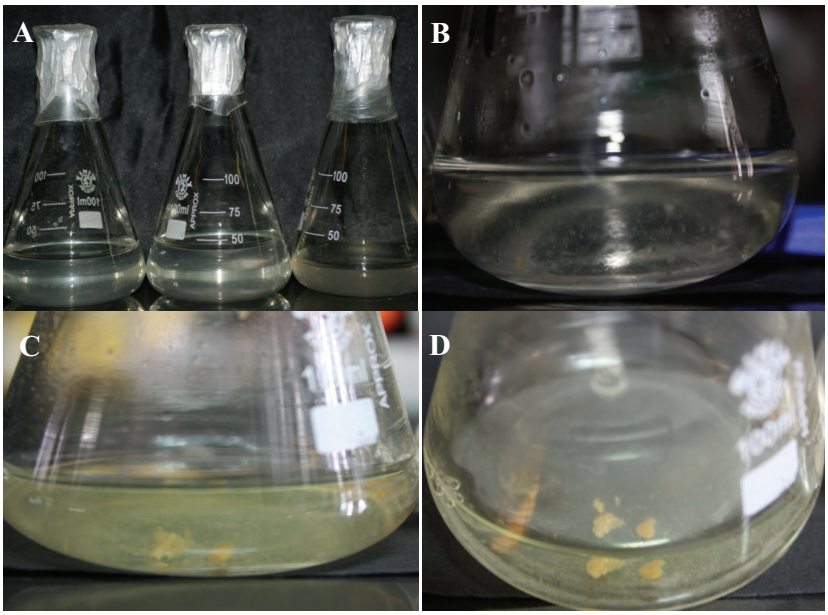

Figure 2. Developmental stage of protoplast in culture suspension A) culture suspension contain release protoplast B) cell proliferation and growth after two days and turbid suspension medium C) Formation of cell masses After 14 days D) Cell mass enlargement and callus formation after 20 days of culture.

In this study, plants were isolated and regenerated from *Fritillaria imperialis* L. protoplasts. Likewise, 0.2 gr 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 fact, this study was directly concerned with the enzyme-substrate relationship (Bodansky, 1954).

This 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). Another important factor for protoplast culture is the culture system.

In these experiments protoplasts were cultured either in liquid and solid MS medium comprising 1×10^5 and 1×10^5 protoplasts/ml. Division of protoplasts obtained in liquid MS medium at optimal density was 1.26×10^6 protoplasts/ml. 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.

In contrast to published data (Kisaka *et al*., 1998) the same gelrite was successfully used for protoplast culture. There were some reports that agarose and phytagel have been used to improve protoplast culture in *Medicago sp*. and *Garcinia atroviridis* Griff., respectively (Techato, 1997)

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 of development of microcalli (Horita et al., 2002).

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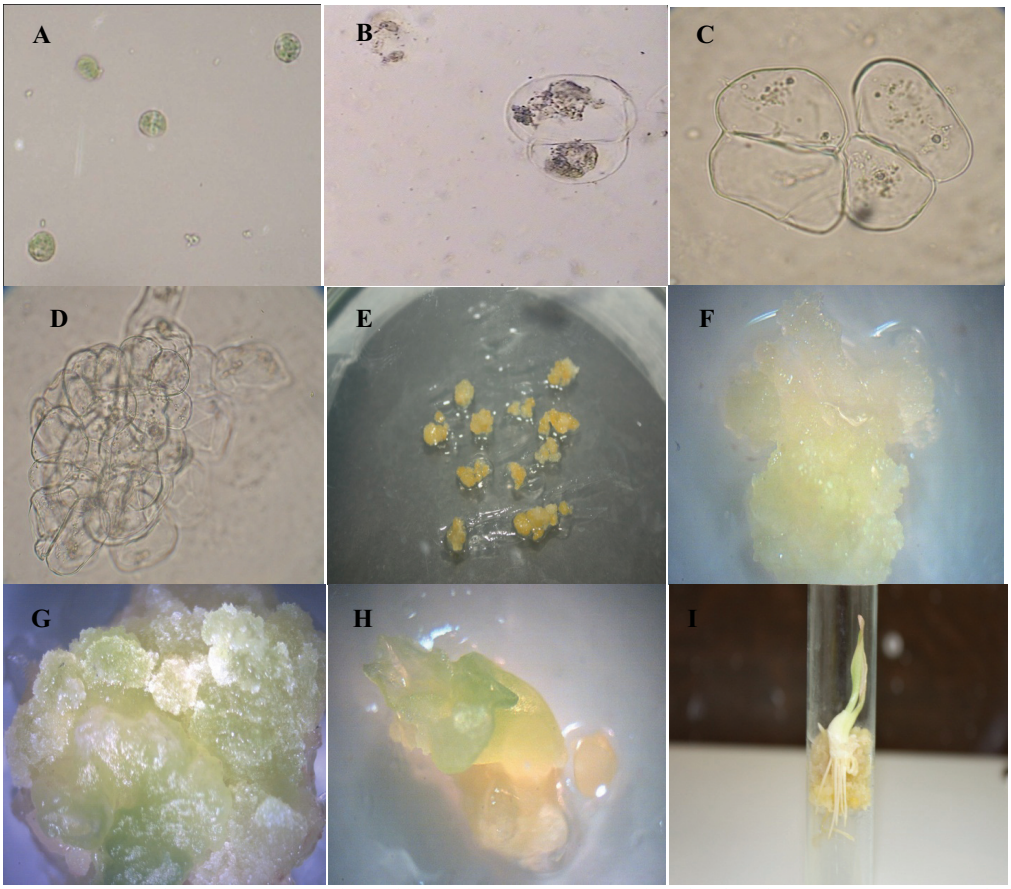

Figure 3. General overview of protoplast culture and regeneration procedure developed for *F. imperialis.* A) Isolated protoplasts from callus. B) First division after 48 hr of culture C) second division after 4 days of culture D, E) colony formation after 3 weeks of culture F) Plate of cell suspension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

The number of microcalli we obtained was close to those obtained in earlier studies in banana (Assani et al., 2001). However, the obtained calli did not develop into plants in our study. Auxin is involved in cell division and callus formation. 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 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 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 gr 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}. Shoots were transferred to hormone free MS-medium to induce root formation. 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 (Pongchawee et al., 2006). According to Tamura *et al*. (1992) report, high concentration of glucose (0.5M) is followed the best outcome for protoplast culture. They also proved that, addition of Zeatin (1 mM) and NAA (10 mM) gives the normal size of the colonies formed. Changed protoplast culture medium to 5.4 mM NAA and 2.3 mM Zeatin was suitable for protoplast regeneration. So, that was the appropriate density of cells in the medium (Tian *et al*., 1999). Also, cultured 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 (Saker et al., 1999).

CONCLUSIONS

The best treatment for isolation of protoplast, growth, division cells, 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.

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