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# Plant regeneration by organogenesis from bulbous explants in *Fritillaria imperialis* L., a wild rare ornamental species at the risk of extinction

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*Key words*: crown imperial, micropropagation, plant growth regulators, threatened ornamentals, tissue culture.



**Abstract: *Fritillaria imperialis* L. (Liliaceae) is a rare and endangered ornamental plant grown in mountain regions and Zagros altitudes, Ilam province, Iran. This species is in danger of extinction due to invasive collection. Plant regeneration was done by organogenesis from bulb scales as explants cultured on Murashige and Skoog (MS) media fortified with different concentrations of kinetin (KIN, 0.00, 0.50, 1.00 and 2.00 mg $l^{-1}$) and α-naphthaleneacetic acid (NAA, 0.00, 0.50, 1.00 and 2.00 mg $l^{-1}$), either individually or in combination. The largest number of leaf (3.80), root (5.86) and callus (8.16) per explant was regenerated on the medium containing 0.50 mg $l^{-1}$ KIN and 1.00 mg $l^{-1}$ NAA. Maximum viability percentage (96.66%) was obtained in medium supplemented with 1.00 mg $l^{-1}$ KIN. *In vitro* regenerated plantlets were cultivated in plastic pots containing peat moss and perlite (1:1). The plantlets were successfully acclimatized in an adaptation greenhouse with a survival rate of 95% exhibiting normal developmental patterns.**

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**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

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## 1. Introduction

*Fritillaria* (Liliaceae) is a genus of about 100 species of bulbous perennials found in a range of habitats, from woodland to open meadows and high screes, distributed throughout the temperate regions of the N. hemisphere, particularly the Mediterranean, S.W. Asia and W. North America. Each bulb of *Fritillaria* has 2 or more scales, and sometime abundant basal bulblets. *Fritillaria imperialis* L. (crown imperial or tears of Mary) is a perennial plant with high medicinal and ornamental importance (Wang *et al*., 2005). The 14 important species of *F. imperialis* L. are native to Iran (De Hertogh and Le Nard, 1993). Wild populations of *F. imperialis* are mostly found in high altitudes (>2,000 m) of western parts of Iran, particularly in three provinces, Chahar Mahal-va-Bakhtiari, Kohkyluyeh-va-Bouyrahmad and Ilam. The leaves are usually lance-shaped or linear and the flowers, borne in spring or early summer, are usually pendulous and solitary, or in terminal racemes or umbels, and have 6 tepals. *F. imperialis*

L. has been used either as pot plant for designing landscape or cut flower. Thanks to attractive red and yellow flowers, this plant reveals a great commercial potential (De Hertogh and Le Nard, 1993). The bulbs of *F. imperialis* contain alkaloids, non-alkaloid and high starch content (Li *et al*., 2000; Wang *et al*., 2005).

In Iran, wild populations of two important species, *F. imperialis* and *F. persica*, are at the risk of extinction, because of many harvesting, lack of protecting rules, changing the pastures to dry farmlands, and pest and pathogens invasions. The natural proliferation rate of *Fritillaria* is relatively low that hampers the large-scale cultivation of this plant. *F. imperialis* cannot efficiently propagate by traditional methods such as cutting, bulb scale and seed, because of small numbers of scales (3-5) per bulb and restricted amount of meristematic cells (De Hertogh and Le Nard, 1993). Plant proliferation takes 5-7 years through the seed, also seeds have physiological dormancy. In addition, seedlings are weak, survival rate and growth are low, and produced plants by seed are not true-to-type; because of cross-pollinate nature of *Fritillaria* (De Hertogh and Le Nard, 1993; Baskin and Baskin, 2004). Limited availability of bulblets from nature is another problem. These limitations suggest the need to develop alternative propagation methods for commercial production of these elite species.

The application of biotechnology especially *in vitro* proliferation is a suitable method for reproduction of rare and endangered species with difficult propagation and mass production of valuable genotypes (Vetchinkina *et al*., 2012). This method is becoming increasingly important for conservation of rare and endangered plant species (Almeida *et al*., 2005). *In vitro* propagation is an effectively alternative means for rapid multiplication of species, in which conventional methods have problems and limitations. Tissue culture using bulb scale segments, the most commonly used explants in tissue culture of bulbous plants including Liliaceae family (Mirici *et al*., 2005), and other explants such as foliar and flower explants has been reported for some cultivars of *F. imperialis* (Paek and Murthy, 2002).

Two basic morphogenetic ways leading to the regeneration of the whole plant from somatic tissues are organogenesis and embryogenesis. Both ways of morphogenesis can occur as direct (without passing callus phase) and indirect (with passing callus phase). The wild population of *F. imperialis* is highly heterozygote and non-uniform, because of its self-incompatibility nature. There are a few reports on direct and indirect organogenesis in *F. imperialis* (Witomska and Lukaszewska, 1997; Witomska, 2000; Paek and Murthy, 2002; Subotić *et al*., 2010; Kizil and Khawar, 2014). This paper describes a protocol for rapid *in vitro* multiplication of *F. imperialis* L. by bulb scales as explant and KIN and NAA as plant growth regulators (PGRs) that could be helpful for large-scale production for field culture.

## 2. Materials and Methods

The bulbs of *Fritillaria imperialis* L. were harvested from natural habitat (mountain regions and Zagros altitudes, Ilam province, Iran) (Figs. 1, 2A) and used as the starting material for the establishment of *in vitro* culture. Bulbs were transferred to the Plant Tissue Culture and Biotechnology Laboratory, Amol, Mazandaran Province located in the northern part of Iran. In laboratory, bulbs (Fig. 2B) were washed under running tap water (Fig. 2C) for half an hour to remove mud and dirt. The bulbs were put into a vessel filled with water and a few drops of dish-washing liquid for 10 min (Fig. 2D). Then, these were washed in running tap water for half an hour, again. Cleaned bulbs were decontaminated with a fungicide (0.10 mg $l^{-1}$ benomile + carbendazim, rural T.S.) for 20 min followed by once washing for 10 min. in distilled water. The bulbs were sterilized by immersing in 0.01 g $l^{-1}$ mercuric chloride (HgCl) for 15 min with continuous stirring using magnetic stirrer, then by 20% sodium hypochlorite (NaOCl) solution (commercial bleach) for 15 min followed by three times rinsing (each for 10 min.) in sterile distilled water and finally

Fig. 1 - *Fritillaria imperialis* L. growing at its natural habitat (mountain regions and Zagros altitudes, Ilam province, Iran).

the bulbs were dipped in 70% ethanol for 60 sec. The scales were rinsed in sterile distilled water for three times.

Dried bulbs (by placing on paper for 5 min.) were vertically cut into 10 × 10 mm under aseptic conditions to obtain twin scales as explants to induce callus and shoots for *in vitro* propagation (Fig. 2E). Outer scales directly in contact with disinfectant during sterilization were removed before obtaining double scale explants (Fig. 2F).

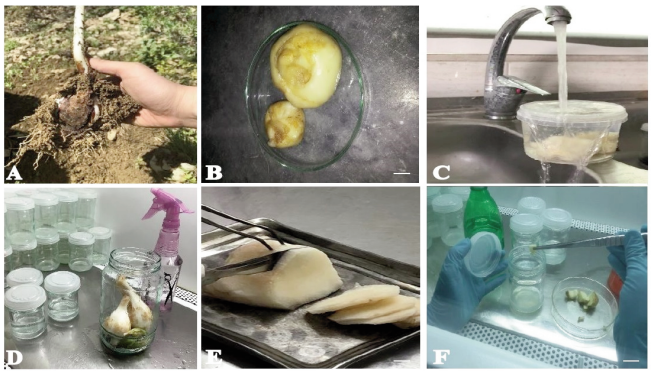


Fig. 2 - Sterilization of bulbs and preparation of scales explants. A) *Fritillaria imperialis* L. eradicated from the soil. B) Separated bulbs. C) Washing the bulbs under running tap water. D) Bulbs ready for sterilization. E) To cut cleaned bulbs as slices (scales). F) Cultivation of scales as explant into the culture vessels (scale bar = 10 mm).

The medium used was MS (Murashige and Skoog, 1962) with 3.00% sucrose and 0.80% Agar-agar. The pH of the medium was adjusted to 5.80. All media were autoclaved at 104.00 kPa and 121°C for 20 min. The scales were cultured on media fortified with NAA and KIN (0.00, 0.50, 1.00 and 2.00 mg $l^{-1}$ from each) to induce shoots from bulb scales. Callus was formed on bulb scales cultured in media containing PGRs. *In vitro* regenerated shoots were grown in these media to find the suitable regeneration potential for shoot and root production. All experiments were performed in 280 ml-jam glasses (6 cm diameter × 10 cm height) and each glass contained 50 ml medium. All cultures were incubated at 24-26°C, 70-80% RH under light intensity of 50 µmol $m^{-2}$ $s^{-1}$ from white fluorescent lamps with 16 h light photoperiod per day. Data were recorded after 65 days of culturing. The parameters: leaf length, leaf diameter, leaf number, root length, root number, callus number and viability percentage were assessed.

*In vitro* produced plantlets were taken out from culture vessels and washed thoroughly under running tap water to remove adherent nutrient and transferred to plastic pots containing peat moss and perlite (1:1). All the pots were then transferred to the adaptation greenhouse with temperature of 24±2°C to 20±2°C day/night, light intensity of 8000 Lux, RH of 80-85% and 14-h photoperiod for acclimatization. After 30 days all plantlets were acclimatized.

The experiments were carried out in a completely randomized design with three replicates per treatment and four scales per replicate (totally; 192 explants). PGRs-free MS medium is used as control in the experiments. The results are expressed as mean ± SD of the experiments. Data pertaining to plantlets growth and development were subjected to analysis of variance (ANOVA) and means were compared by the LSD test at $P < 0.05$ using the SPSS ver. 17 (SPSS Inc., USA).

## 3. Results

The twin bulb scale explants (10×10 mm) of *F. imperialis* L. cultured on MS media containing different concentrations of KIN and NAA showed variation in the frequency of callus formation, shoot regeneration and root formation (Fig. 3, Tables 1-4).

*Leaf induction and proliferation*

Callus was induced on bulb scales (Fig. 3A). Leaves were produced from callus through indirect organogenesis (Figs. 3B, C). The minimum leaf length (1.26 cm) from twin scale explants was measured on MS

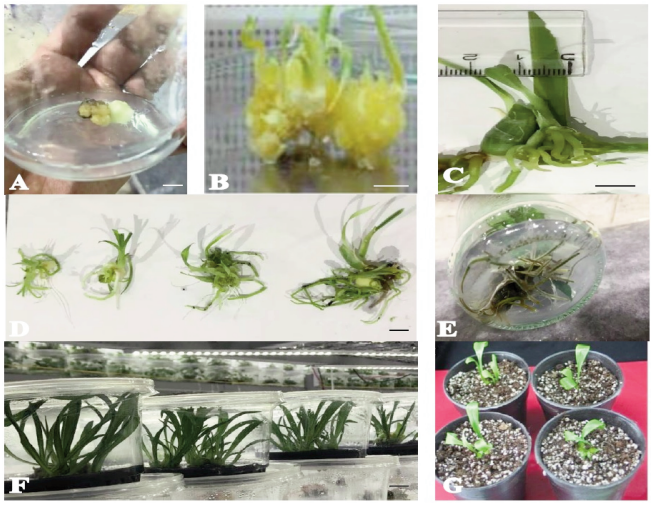


Fig. 3 - Micropropagation process of *Fritillaria imperialis* L. A) Callus production on bulb scales. B) Leaves produced from callus by indirect organogenesis. C) Developed leaves before planting in greenhouse condition. D) Regenerated plantlets from callus on media containing different concentrations of KIN and NAA. E) Rooted plantlets. F) Regenerated leaves in media supplemented with different concentrations of KIN and NAA. G) Acclimatization of plantlets grown into the pots filled with peat moss and perlite (1:1) in an adaptation greenhouse (scale bar = 10 mm).

medium without PGRs (Table 4). The maximum number of 3.16 leaves per explant on twin scale explants was measured on MS medium containing 1.00 mg $l^{-1}$ KIN and 0.50 mg $l^{-1}$ NAA (Figs. 3D, F). Differences of leaf length in samples grown under different concentrations of KIN and KIN in combination with NAA were significant (p≤0.01) (Table 1). No significant difference was seen between NAA and leaf length. There was no any positive effect between increasing leaf length and increasing KIN and NAA concentrations (Tables 2-4). Among all concentrations of KIN used singularly, maximum and minimum leaf length (2.41 and 1.80 cm, respectively) was induced in bulbs treated with 1.00 mg $l^{-1}$ and control (Table 2). On the other hand, among all concentrations of NAA used as singular PGR, maximum and minimum leaf length (2.24 and 1.85 cm, respectively) was induced in bulbs treated with 2.00 and 1.00 mg $l^{-1}$ (Table 3). There was no statistically significant difference among different concentrations of KIN + NAA and leaf diameter (Table 1).

The data clearly show that leaf number is strongly affected by treatments of KIN (p≤0.01), NAA (p≤0.01) and KIN + NAA (p≤0.01) (Table 1). The largest number of leaf (3.80 per explant) was calculated in bulbs grown on medium enriched with 0.50 mg $l^{-1}$ KIN along with 1.00 mg $l^{-1}$ NAA (Table 4; Figs. 3D, F). The smallest number of leaf (1.66 per explant) was obtained in bulbs grown on medium with 2.00 mg $l^{-1}$ KIN along with 1.00 mg $l^{-1}$ NAA. All treatments containing 2.00 mg $l^{-1}$ KIN in combination with all concentrations of NAA produced less than 2.00 leaves per explant (Table 4). Of all concentrations of KIN, the largest and smallest number of leaf (3.33 and 1.85, respectively) was induced in explants grown on medium enriched with 0.50 and 2.00 mg $l^{-1}$ (Table 2). Also, differences in leaf number between all concentrations of NAA were not noticeable.

*Root induction and growth*

The maximum average of the root length per

Table 1 - Analysis of variance of the effect of different concentrations of KIN and NAA on measured characters of *Fritillaria imperialis* L.

| Source of variations | df | Mean of Squares | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Leaf length | Leaf diameter | Leaf number | Root length | Root number | Callus number | Viability percentage |
| KIN | 3 | 0.877 ** | 0.020 NS | 4.580 ** | 4.820 ** | 4.201 ** | 7.440 ** | 413.80 ** |
| NAA | 3 | 0.379 NS | 0.041 * | 0.690 ** | 1.023 ** | 1.397 NS | 2.087 * | 113.80 NS |
| KIN × NAA | 9 | 0.686 ** | 0.030 NS | 0.356 ** | 1.059 ** | 1.815 ** | 5.270 ** | 67.50 NS |
| Error | 32 | 0.216 | 0.014 | 0.099 | 0.226 | 0.554 | 0.636 | 50.00 |
| cv (%) | | 22.40 | 15.80 | 12.10 | 12.90 | 18.03 | 18.00 | 8.35 |

*, ** = Significant at the 0.05 and 0.01 probability level, respectively, NS= Not significant at p=0.05.

Table 2 - Mean comparison of the effect of different concentrations of KIN on measured characters of *Fritillaria imperialis* L.

| KIN (mg $l^{-1}$) | Leaf length (cm) | Leaf diameter (mm) | Leaf number | Root length (cm) | Root number | Callus number | Viability (%) |
|---|---|---|---|---|---|---|---|
| 0.00 | 1.800 b | 0.7666 a | 2.750 b | 2.833 c | 3.483 b | 3.491 b | 78.33 b |
| 0.50 | 1.933 b | 0.8083 a | 3.333 a | 3.700 b | 4.866 a | 5.266 a | 89.16 a |
| 1.00 | 2.416 a | 0.7083 a | 2.450 c | 4.375 a | 4.341 a | 4.858 a | 90.00 a |
| 2.00 | 2.158 ab | 0.7666 a | 1.850 d | 3.766 b | 3.616 b | 4.116 b | 80.83 b |

Means with different letters on the same column are significantly different (p<0.05) based on LSD test.

Table 3 - Mean comparison of the effect of different concentrations of NAA on measured characters of *Fritillaria imperialis* L.

| NAA (mg $l^{-1}$) | Leaf length (cm) | Leaf diameter (mm) | Leaf number | Root length (cm) | Root number | Callus number | Viability (%) |
|---|---|---|---|---|---|---|---|
| 0.00 | 2.008 a | 0.750 ab | 2.80 a | 3.266 b | 3.74 b | 4.458 ab | 85.00 ab |
| 0.50 | 2.200 a | 0.700 b | 2.31 b | 3.791 a | 4.33 ab | 4.333 ab | 80.83 b |
| 1.00 | 1.858 a | 0.758 ab | 2.79 a | 3.950 a | 4.48 a | 4.975 a | 84.16 ab |
| 2.00 | 2.241 a | 0.841 a | 2.47 b | 3.666 a | 3.95 ab | 3.966 b | 88.33 a |

Means with different letters on the same column are significantly different (p<0.05) based on LSD test.

explant (5.50 cm) was calculated with treatment of 1.00 mg $l^{-1}$ KIN and 1.00 mg $l^{-1}$ NAA (Table 4). The minimum average of the root length per explant (2.30 cm) was measured with treatment without KIN and NAA (control). Concerning the root length induced by various concentrations of KIN, the maximum and minimum length (4.37 and 2.83 cm) was obtained in media containing 1.00 mg $l^{-1}$ and control, respectively (Table 2). Concerning the root length induced by various concentrations of NAA, the maximum and minimum length (3.95 and 3.26 cm) was obtained in media containing 1.00 mg $l^{-1}$ and control, respectively (Table 3). There was statistically significant difference among different concentrations of KIN, NAA also KIN in combination with NAA and root length ($p \leq 0.01$).

KIN in combination with NAA was superior in induction of root and on the medium fortified with KIN (0.50 and 1.00 mg $l^{-1}$) + NAA (0.50 and 1.00 mg $l^{-1}$) optimal of over than 5 roots were produced per explant on MS medium (Table 4). The largest numbers of roots (5.86 per explant) was formed in MS medium supplemented with 0.50 mg $l^{-1}$ KIN plus 1.00 mg $l^{-1}$ NAA (Table 4; Fig. 3E). Bulbs scales cultured on MS medium without any PGRs produced least roots (2.83). The root number (5.40 and 5.00 per explant) in media containing 0.50 mg $l^{-1}$ KIN plus 0.50 mg $l^{-1}$ NAA and 1.00 mg $l^{-1}$ KIN plus 0.50 mg $l^{-1}$ NAA was proper (Table 4). Concerning the root number produced using various levels of KIN and NAA, each one as singularly; the largest and smallest number (4.86 and 3.48) was counted in media fortified with 0.50 mg $l^{-1}$ KIN and control, respectively (Tables 2, 3). Analysis of variance (ANOVA) test demonstrated that concentrations of KIN and KIN in combination with NAA were significant with respect to number of roots produced from scale sections (Table 1).

*Callus induction*

Current investigation demonstrated variable frequency of callus induction on bulbs scales at various concentrations of KIN and NAA in the culture medium. Callus was emerged after 30 days of culture (Fig. 3A). Callus formation started at the margins of bulbs scales. Callus induction was evident in response to the presence of both KIN and NAA. Maximum (8.16) and minimum (3.00) callus number per explant was observed on explants cultured on medium supplemented with 0.50 mg $l^{-1}$ KIN plus 1.00 mg $l^{-1}$ NAA and medium without PGRs (control), respectively (Table 4).

*Viability percentage*

Viability percentage of the bulbs scales in the media was changed significantly with the use of different PGRs (Table 4). Viability percentage of the bulbs scales in MS medium supplemented with 1.00 mg $l^{-1}$ KIN without NAA (96.66%) was the maximum. Viability percentage in medium supplemented with 1.00 mg $l^{-1}$ NAA without KIN (73.33%) was the minimum (Table 4). Statistically significant differences were recovered between the means for viability percentage and KIN (Table 1).

Table 4 - Mean comparison of the effect of different concentrations of KIN and NAA on measured characters of *Fritillaria imperialis* L.

| KIN (mg $l^{-1}$) | NAA (mg $l^{-1}$) | Leaf length (cm) | Leaf diameter (mm) | Leaf number | Root length (cm) | Root number | Callus number | Viability (%) |
|---|---|---|---|---|---|---|---|---|
| 0.00 | 0.00 | 1.266 ±0.208 d | 0.733±0.0289 abcd | 2.700±0.000 cd | 2.300± 0.200 f | 2.836±0.252 h | 3.000±0.854 e | 76.66±2.887 de |
| 0.00 | 0.50 | 1.366±0.208 d | 0.533±0.0577 d | 2.266±0.058 ef | 2.933± 0.115 f | 3.233±0.153 e-h | 4.000±0.854 cde | 80.00±0.000 c-e |
| 0.00 | 1.00 | 2.200±0.781 bc | 0.900±0.1000 a | 3.233±0.252 c | 2.766±0.208 f | 3.700±0.200 d-h | 3.333±0.473 e | 73.33±5.774 e |
| 0.00 | 2.00 | 2.366±0.513 bc | 0.900±0.1000 a | 2.500±0.100 e | 2.833f ± 0.153 | 4.733±0.451 | 3.033±0.462 e | 83.33±5.774 b-e |
| 0.50 | 0.00 | 1.933±0.493 bcd | 0.800±0.1000 abc | 3.700±0.000 ab | 2.933±0.058 f | 4.400±1.015 b-f | 3.133±0.666 e | 90.00±10.000 abc |
| 0.50 | 0.50 | 1.933±0.153 bcd | 0.766±0.0577 abc | 2.500±0.200 e | 4.666±0.058 b | 5.400±0.656 ab | 5.033±1.514 bc | 86.66±15.275 abcd |
| 0.50 | 1.00 | 1.700±0.458 cd | 0.800±0.1000 abc | 3.800±0.100 a | 3.366±0.153 ef | 5.866±1.422 a | 8.166±0.702 a | 86.66±5.774 a |
| 0.50 | 2.00 | 2.166±0.321 bc | 0.866±0.0577 ab | 3.333±0.153 bc | 3.833±0.289 cde | 3.800±0.781 c-h | 4.733±1.106 cd | 93.33±11.547 ab |
| 1.00 | 0.00 | 2.333±0.802 bc | 0.666±0.2082 bcd | 2.533±0.603 e | 3.466±0.208 def | 3.333±0.473 f-h | 6.233±0.850 b | 96.66±5.774 a |
| 1.00 | 0.50 | 3.166±0.416 a | 0.666±0.1528 bcd | 2.566±0.473 de | 4.300±0.100 bc | 5.000±1.803 abc | 3.933±0.153 cde | 80.00±5.000 c-e |
| 1.00 | 1.00 | 1.700±0.458 cd | 0.700±0.1732 abcd | 2.466±0.751 e | 5.500±0.854 a | 4.666±0.777 a-e | 4.866±0.603 bcd | 90.00±5.000 abc |
| 1.00 | 2.00 | 2.466±0.252 abc | 0.800±0.2000 abc | 2.233±0.451 ef | 4.233±0.451 bc | 4.366±0.493 b-f | 4.400±0.721 cde | 93.33±2.887 ab |
| 2.00 | 0.00 | 2.500±0.721 ab | 0.800±0.1732 abc | 1.966±0.058 fg | 3.866±0.862 cde | 3.766±0.115 d-h | 4.86±0.635 bcd | 76.66±5.774 de |
| 2.00 | 0.50 | 2.333±0.153 bc | 0.833±0.1155 abc | 1.933±0.115 fg | 3.266±0.666 ef | 4.100±0.173 c-g | 4.366±0.850 cde | 76.66±5.774 de |
| 2.00 | 1.00 | 1.833±0.351 bcd | 0.633±0.0577 cd | 1.666±0.231 g | 4.166±0.850 bcd | 3.700d±0.265 -h | 3.533±0.666 de | 86.66±5.774 a-d |
| 2.00 | 2.00 | 1.966±0.416 a-d | 0.800±0.0000 abc | 1.833±0.153 fg | 3.766±0.702 cde | 2.900±0.100 gh | 3.700±0.800 cde | 83.33±5.774 b-e |

Means with different letters on the same column are significantly different ($p<0.05$) based on LSD test (Mean ± SD).

Plantlets were successfully acclimatized in an adaptation greenhouse and recorded 95% survival rate after 30 days in pots filled with perlite: peat moss (1:1 v/v) (Fig. 3G). There were no visual morphological abnormalities in the micropropagated plantlets.

## 4. Discussion and Conclusions

*Fritillaria* is a rare and critically threatened genus due to the large-scale eradication, irregular grazing and lack of protecting rules. This plant reveals a great commercial potential, therefore, the plants in danger of extinction, like *Fritillaria*, should be protected. *In vitro* propagation is an effective method for conservation and rapid multiplication of the species in danger of extinction like the members of Liliaceae family including *Fritillaria* due to limitations in conventional methods of propagation.

Various factors influence the process of micropropagation from bulb scales and *in vitro* regenerated bulblet explants, especially type, concentration and combination of PGRs particularly auxins and cytokinins. Optimal selection of PGRs is especially important when the amount of the original plant material of rare and endangered plant species is limited (Kulkhanova *et al*., 2015).

For *F. imperialis*, *in vitro* propagation is carried out by bulb scales using mainly NAA, IAA and BA (Witomska and Lukaszewska, 1997; Lukaszewska *et al*., 1998; Witomska *et al*., 1998; Mohammadi-Dehcheshmeh *et al*., 2006; Petrić *et al*., 2013). For most members of the genus *Fritillaria*, micropropagation is done by bulb scales using mainly BA, BAP, TDZ, NAA, IAA and 2,4-D (Petrić *et al*., 2013; Kulkhanova *et al*., 2015). The concentrations of 0.10-4.00 mg $l^{-1}$ of NAA in combination with 0.10-2.00 mg $l^{-1}$ KIN has been applied using bulb scales for *in vitro* propagation of some species of the genus *Fritillaria*, such as *F. anhuiensis* (Xue *et al*., 2008), *F. camtschatcensis* (Okawa and Nishino, 2000), *F. roylei Hook* (Joshi *et al*., 2007), *F. thunbergii* (Seon *et al*., 1999), and *F. ussuriensis* (Sun and Wang, 1991), not for *F. imperialis*. NAA is the most effective auxin in inducing the *in vitro* formation of bulblets from the segments of bulb scales of *F. sonnikovae*, and the maximal regeneration was obtained combining 1.62 µM NAA and 4.65 µM KIN also 5.00 µM BAP and 2.00 µM NAA (Kulkhanova *et al*., 2015). The use of BAP in combination with IAA was effective for micropropagation of *F. unibracteata* (Gao *et al*., 1999). In our study, the use of 0.50 mg $l^{-1}$ KIN and 1.00 mg $l^{-1}$ NAA was useful for shoot and root production through indirect organogenesis.

Cytokinins are generally known to promote the formation of buds in many excised and *in vitro* tissue cultured organs. The medium containing 2.20 µM BA was most effective for shoot formation on bulb scales of *Lilium longiflorum* (Han *et al*., 2004). Similar results were also reported by others (Naik and Nayak, 2005). Bulblets regeneration and shoot multiplication on bulb scale explants were observed in some Liliaceae members, like *F. thunbergii* and *Ornithogalum ulophyllum* (Paek and Murthy, 2002; Ozel *et al*., 2008). Ipek *et al*. (2006) applied different concentrations of BAP, NAA and KIN to obtain bulblets from immature embryos of *Ornithogalum platyphyllum*. Naik and Nayak (2005) induced direct induction of bulblets on the bulb scales grown on the MS media enriched with 1.00 mg $l^{-1}$ NAA and 2.00 mg $l^{-1}$ BA. Naik and Nayak (2005) showed a plant regeneration procedure in *Ornithogalum virens* through direct shoot bud formation and indirect organogenesis using bulb scale as explant cultured on MS medium containing 1.00 mg $l^{-1}$ NAA and 2.00 mg $l^{-1}$ BA. Paek and Murthy (2002) revealed that the maximum bulblet regeneration and leaf production in *F. thunbergii* using bulb scale segments as explants were obtained in MS medium supplemented with a combination of KIN and NAA. Current investigation is consistent with this finding. Our study showed the same importance of KIN and NAA for shoot regeneration. In agreement with us, study of Kukulczanka *et al*. (1989) on *F. meleagris* L. demonstrated that the highest percentage of the regenerating explants, was obtained in case of joint action of cytokinin and auxin. In *F. aurea* Schott, increased TDZ concentrations increased leaf number. The highest regeneration rate was obtained from combination of cytokinin and auxins (Kizil *et al*., 2016). In *F. imperialis*, the number of regenerated shoots was the highest on MS medium supplemented with 0.50 mg $l^{-1}$ TDZ and the number of roots was the highest on MS medium supplemented with 0.20 mg $l^{-1}$ NAA (Rahimi *et al*., 2014).

We observed variable frequency of callus induction at various concentrations of KIN-NAA in the culture medium. Similar findings were reported by others using BAP-NAA (Nayak and Sen, 1995; Naik and Nayak, 2005; Ozel *et al*., 2008). Malabadi and Van Staden (2004) induced shoot bulblets and embryogenic calli in *Ornithogalum longibracteatum* on medium containing KIN. In the study of Çakmak *et al*. (2016) on *F. persica*, all explants including bulb scales showed competency

to regenerate callus and bulblets using 2,4-D and KIN, or TDZ. Bulblet explants responded fairly well to a higher level of TDZ in order to obtain maximum shoot regeneration. Callus induction was evident in response to high TDZ concentration. Current study showed that the maximum root formation was obtained on medium containing both KIN and NAA. Similar to our finding, Ozel *et al*. (2008) successfully rooted bulblets regenerated on 2.00 mg $l^{-1}$ BAP and 0.50 mg $l^{-1}$ NAA. In *F. aurea* Schott, maximum rooting was noted on 0.50 mg $l^{-1}$ IBA (Kizil *et al*., 2016).

In conclusion, *F. imperialis*, bulbous cultivated as ornamental cut flower and garden plant, has important also as medicinal plant and it is at the risk of extinction. Traditional propagation is limited, because production of bulbs is poor and seeds have a low germination rate. Therefore, *in vitro* propagation by direct and indirect organogenesis and embryogenesis is a proper approach. The success of these procedures highly depends on type and concentrations of auxins and cytokinins applied in culture medium, singular or in combination. In our study, 0.50 mg $l^{-1}$ KIN along with 1.00 mg $l^{-1}$ NAA successfully induced leaf and root formation.

## References


ALMEIDA R., GONCALVES, S., ROMANO A., 2005 - In vitro *micropropagation of endangered* Rhododendron ponticum *L. sub sp. Baeticum (Boissier and Reuter) Handel-Mazzetti*. - Biodiver. Conser., 14: 1059-1069.

BASKIN J.M., BASKIN C.C., 2004 - *A classification system for seed dormancy*. - Seed Sci. Res., 14: 1-16.

ÇAKMAK D., KARAOĞLU C., AASIM M., SANCAK C., ÖZCAN S., 2016 - *Advancement in protocol for in vitro seed germination, regeneration, bulblet maturation, and acclimatization of* Fritillaria persica*.* - Turk. J. Biol., 40: 878-888.

DE HERTOGH A., LE NARD M., 1993 - *General chapter on summer flowering bulbs,* pp. 741-774. - In: DE HERTOGH A., and M. LE NARD (eds.). *The Physiology of flower bulbs: A comprehensive treatise on the physiology and utilization of ornamental flowering bulbous and tuberous plants*. Elsevier, Amsterdam, The Netherlands, pp. 811.

GAO S., ZHU D., CAI Z., JIANG Y., XU D., 1999 - *Organ culture of a precious Chinese medicinal plant*-Fritillaria unibracteata. - Plant Cell Tiss. Org. Cult., 59: 197-201.

HAN B.H., YU H.J., YAE B.W., PEAK K.Y., 2004 - In vitro *micropropagation of* Lilium longiflorum *'Georgia' by shoot formation as influenced by addition of liquid medium*. - Sci. Hortic., 103: 39-49.

IPEK A., COCU S., URANBEY S., KAYA M.D., GURLEK D., AKDOGAN G., HAKYEMEZ, H., SANCAK C., OZCAN S., 2006 - In vitro *bulblet proliferation in* Ornithogalum platyphyllum. - Proceedings of the International Symposium Agro environ. Agricultural Constraints in the Soil and Plant Atmosphere Continuum, 4-7 September, Ghent University, Belgium, pp. 301-303.

JOSHI S.K., DHAR U., ANDOLA H.C., 2007 - In vitro *bulblet regeneration and evaluation of* Fritillaria roylei *Hook. A high value medicinal herb of the Himalaya*. - Acta Horticulturae, 756: 75-84.

KIZIL S., KHAWAR K.M., 2014 - *The effects of plant growth regulators and incubation temperatures on germination and bulb formation of* Fritillaria persica *L*. - Propag. Ornam. Plants, 14: 133-138.

KIZIL S., SOGUT T., SESIZ U., KHAWAR KH.M., 2016 - *Accelerated micropropagation of endemic* Fritillaria aurea *Schott*. - Sci. Bulletin. Series F. Biotechnologies, 20: 99-104.

KUKULCZANKA K., KROMER K., CZASTKA B., 1989 - *Propagation of* Fritillaria meleagris *L. through tissue culture*. - Acta Horticulturae, 251: 147-153.

KULKHANOVA D.S., ERST A.A., NOVIKOVA T.I., 2015 - In vitro *regeneration from bulbous scales of* Fritillaria sonnikovae*, an endemic species*. - Russ. J. Dev. Biol., 46(4): 215-221.

LI S.L., LI P., LIN G., CHAN S.W., HO Y.P., 2000 - *Simultaneous determination of seven major isosteroidal alkaloids in bulb of* Fritillaria *by gas chromatography*. - J. Chromatogr., 873: 221-228.

LUKASZEWSKA A.J., WITOMSKA M., BIANCO J., BARTHE P., 1998 - *ABA contents and the regeneration ability of* Fritillaria imperialis *L. cultured* in vitro. - Acta Physiol. Plant., 20(3): 241-244.

MALABADI R.B., VAN STADEN J., 2004 - *Regeneration of* Ornithogalum in vitro. - South Afr. J. Bot., 70: 618-621.

MIRICI S., PARMAKSIZ I., ÖZCAN S., SANCAK C., URANBEY S., SARIHAN E.O., GÜMÜŞCÜ A., GÜRBÜZ B., ARSLAN N., 2005 - *Efficient* in vitro *bulblet regeneration from immature embryos of endangered* Sternbergia fischeriana. - Plant Cell Tiss. Org. Cult., 80: 239-246.

MOHAMMADI-DEHCHESHMEH M., KHALIGHI A., EBRAHIMIE E., SARDARI M., NADERI R., 2006 - *Direct bulblet regeneration from mature embryo: a rapid, efficient and genotype-independent in vitro morphogenesis pathway for preservation of endangered wild populations of* Fritillaria imperialis *and* Fritillaria persica. - HortSci., 41(4): 1066C-1066.

MURASHIGE T., SKOOG F., 1962 - *A revised medium for rapid growth and bio assays with tobacco tissue cultures*. - Physiol. Plant., 15: 473-497.

NAIK P.K., NAYAK S., 2005 - *Different modes of plant regeneration and factors affecting* in vitro *bulblet production in* Ornithogalum virens. - Sci. Asia, 31: 409-414.

NAYAK S., SEN S., 1995 - *Rapid and stable propagation of* Ornithogalum umbellatum *L. in long-term culture*. - Plant Cell Rep., 15: 150-153.

OKAWA M., NISHINO K., 2000 - *Effect of sucrose and poly-*

*ethylene glycol on leaf emergence, rooting and bulblet growth of* Fritillaria camtschatcensis *Ker-Gawl.* In vitro *culture*. - Environ. Control Biol., 38 (2): 99-103.

OZEL C.A., KHAWAR KH.M., KARAMAN S., ATESS M.A., ARSLAN O., 2008 - *Efficient* in vitro *multiplication in* Ornithogalum ulophyllum *Hand.-Mazz. from twin scale explants*. - Sci. Hortic., 116: 109-112.

PAEK K.Y., MURTHY H.N., 2002 - *High frequency of bulblet regeneration from bulb scale sections of* Fritillaria thunbergii. - Plant Cell. Tiss. Org. Cult., 68: 247-252.

PETRIĆ M., JEVREMOVIĆ S., TRIFUNOVIĆ M., TADIĆ V., MILOŠEVIĆ S., DRAGIĆEVIĆ M., SUBOTIC A., 2013 - *The effect of low temperature and $GA_3$ treatments on dormancy breaking and activity of antioxidant enzymes in* Fritillaria meleagris *bulblets cultured* in vitro. - Acta Physiol. Plant, 35: 3223-3236.

RAHIMI M., DANESHVAR M.H., HEIDARI M., AHVAZ I., 2014 - *Propagation and bulb formation of Fritillaria (*Fritillaria imperialis*)* via in vitro *culture*. - Int. J. Plant Animal Environ. Sci., 4: 707-710.

SEON J.H., PAEK K.Y., GAO W.Y., PARK C.H., SUNG N.S., 1999 - *Factors affecting micropropagation of pathogen-free stocks in* Fritillaria thunbergii. - Acta Horticulturae, 502: 333-337.

SUBOTIĆ A., TRIFUNOVIĆ M., JEVREMOVIĆ S., PETRIĆ M., 2010 - *Morphohistological study of direct somatic embryogenesis in endangered species* Frittilaria meleagris. - Biol. Plant, 54 (3): 592-596.

SUN C.S., WANG D.Y., 1991 - Fritillaria *spp. (Fritillary):* In vitro *culture and the regeneration of plants,* pp. 258-269. - In: BAJAJ Y.P.S. (ed.) *Biotechnology in Agriculture and Forestry 15, Medicinal and Aromatic Plants III*. Springer-Verlag, Heidelberg, Germany, pp. 502.

VETCHINKINA E.M., SHIRNINA I.V., SHIRNIN S.Y., MOLKANIVA O.I., 2012 - *Preservation of rare species of plants in genetic collections* in vitro. - Vestn. Balt. Feder. Univ. Im. I. Kanta, 7: 109-118.

WANG S., GAO W., CHEN H., XIAO P., 2005 - *New starches from* Fritillaria *species medicinal plants*. - Carbohyd. Polym., 61: 111-114.

WITOMSKA M., 2000 - *The effect of the bulb storage temperature on the levels of soluble sugars, ABA and cytokinins and the* in vitro *regeneration of* Fritillaria imperialis *L*. - Problem Papers Progress Agric. Sci., 473: 335-341 (in Polish with abstract in English).

WITOMSKA M., LUKASZEWSKA A., 1997 - *Bulblet regeneration* in vitro *from different explants of* Fritillaria imperialis. - Acta Horticulturae, 430: 331-338.

WITOMSKA M., WILK T., LUKASZEWSKA A., 1998 - *Effect of sterilization method on infection level and regeneration* in vitro *of explants from bulbs of* Fritillaria imperialis *L*. - Sci. Papers Ins. Pomol. Floricult., 5: 121-130 (in Polish with abstract in English).

XUE J.P., ZHANG A.M., GENG M.L., MA L., 2008 - *Study on bulblet induction of* Fritillaria anhuiensis in vitro. - China J. Chinese Materia Medica, 33(22): 2603-2606.