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Advancement in protocol for in vitro seed germination, regeneration, bulblet maturation, and acclimatization of *Fritillaria persica*

Derya ÇAKMAK[1], Cuma KARAOĞLU[2], Muhammad AASIM[3], Cengiz SANCAK[4], Sebahattin ÖZCAN[4,*]

[1]Alanya District Directorate, Ministry of Food Agriculture and Livestock, Antalya, Turkey
[2]Central Research Institute for Field Crops, Ministry of Food Agriculture and Livestock, Yenimahalle, Ankara, Turkey
[3]Department of Biotechnology, Faculty of Science, Necmettin Erbakan University, Konya, Turkey
[4]Department of Field Crops, Faculty of Agriculture, Ankara University, Ankara, Turkey

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Abstract: The present study addressed the successful in vitro seed germination, bulblet regeneration, increased bulblet size, hardening, and acclimatization of Persian lily (*Fritillaria persica*). Seed germination rate was recorded as 86.7% and 96.7% after 2 and 3 months of cold treatment at 4 °C, respectively. Bulblet explants taken from a germination experiment were cultured on Murashige and Skoog (MS) medium supplemented with 0.5–2.0 mg/L thidiazuron (TDZ). Maximum bulblet regeneration frequency and bulblets per explants were achieved on a medium containing 2.0 mg/L TDZ. Leaf disc and leaf scale explants isolated from germinated seedlings were also cultured on MS medium containing 2–6 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) with a constant concentration of 0.2 mg/L kinetin. Both explants yielded the highest bulblet regeneration on MS medium containing 2 mg/L 2,4-D and 0.2 mg/L kinetin. A combination of 1.5 g/L agar and 1.6 g/L GELRITE resulted in a maximum bulblet diameter with a higher number of roots. The addition of 10 g/L NaCl to the culture medium resulted in the best root and leaf formation and secondary bulblet regeneration. Exposure of regenerated bulblets to 10 g/L NaCl at 4 °C for 2 months on a medium with 20 g/L sucrose was also found to be beneficial for acclimatization.

Key words: *Fritillaria persica*, micropropagation, secondary bulblets, bulblet size, hardening

1. Introduction

The genus *Fritillaria* (Liliaceae) is an important geophytic taxon with more than 100 species and wide distribution in different climatic zones ranging from southern Europe to the Middle East (Le Nard and De Hertogh, 1993), including Turkey (Bryan, 2002). In Turkey, there are 35 species representing 48 taxa of *Fritillaria*, and it is ranked 4th after *Allium*, *Iris*, and *Crocus*. Most of the *Fritillaria* species in Turkey are endemic. The plants flower during spring and are widely accepted as ornamental plants due to their attractive flowers (Le Nard and De Hertogh, 1993). Besides that, the *Fritillaria* species are used as medicinal plants (Rønsted et al., 2005; Wang et al., 2005) in traditional Chinese medicine (Li et al., 2001) due to a wide array of alkaloids with interesting phytochemical properties.

Persian lily (*Fritillaria persica*) is found mainly in Hatay, Mersin, and Adıyaman provinces of Turkey, as well as in Lebanon, Jordan, and Iran at 700 m to 2800 m above sea level. The bulbs are 3–5 cm in diameter with 2–3 succulent leaves. The plant has 10–25 leaves, which are approximately 15 cm long and 3 cm wide. It bears 7–20 purple bell-shaped flowers, growing in racemose position. The plant can grow up to 20–60 cm in height (Ulug et al., 2010).

Conventional plant production methods currently used for the *Fritillaria* species are seed or bulb propagation. These propagation techniques have certain disadvantages such as very low or unpredictable germination of seeds due to physiological dormancy (Baskin and Baskin, 2004), with weak seedlings, low survival rate, and slow growth, which may take 3–4 years for bulblet maturation (Le Nard and De Hertogh, 1993). Apart from this, a limited availability of bulblets from nature (Subotić et al., 2010) also makes it difficult to get sufficient material to propagate these ornamental species. These limitations suggest the need to develop alternative propagation methods (Ulug et al., 2010) for commercial production of these valuable species.

A number of regeneration protocols have been reported for different *Fritillaria* species by Paek et al. (1996), Sun et al. (1977), Kukulczanka et al. (1988), Gao et al. (1999), Paek and Murthy (2002), Mohammadi-Dehcheshmeh et al. (2006), Subotić et al. (2010), and Kizil and Khawar

* Correspondence: ozcans@ankara.edu.tr

(2014). These regeneration protocols involve the use of immature/mature embryos, bulblets, or bulbous scales explants for most *Fritillaria* species. These explants have certain disadvantages, such as dormancy, which lead to a low germination rate. Furthermore, bulblets or bulbous scales collected from nature are highly contaminated with microorganisms (fungal or bacterial), which hinder the in vitro propagation. Other major problems associated with these in vitro protocols are insufficient bulblet size, maturity, and bulblet dormancy (Langens-Gerrits et al., 2003), which lead to failure of these bulbs to tolerate abiotic stress (Kizil and Khawar, 2014) and ultimately results in unsuccessful acclimatization. Besides that, rooting and hardening of in vitro regenerated *Fritillaria* bulblets is also a challenge for researchers.

To our best knowledge, no study to date has addressed all of the problems mentioned above for any *Fritillaria* species. There is a dire need to develop new regeneration protocols that provide a solution for all the problems in a single study. Therefore, we targeted these problems and present a report for the first time that covers experiments about in vitro germination, bulblet regeneration, increasing bulblet size, hardening, and acclimatization in this study. The results of the present study will be helpful for in vitro propagation of other *Fritillaria* species or bulbous plants that can be used for commercial production.

2. Material and methods

2.1. Plant material and sterilization

Fruits of *F. persica* containing seeds were collected from an experimental field area of the Department of Field Crops, Faculty of Agriculture, Ankara University, Ankara, Turkey. Thereafter, the seeds were subjected to surface sterilization using 70% (v/v) ethanol and 2.5% NaOCl (commercial bleach). The sterilization was done for 20 min followed by rinsing 3 times with distilled sterilized water for 5 min each.

2.2. Seed germination

Surface sterilized seeds were placed on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) in Magenta GA7 vessels for in vitro germination and incubated at three different temperatures of 4, 16, and 24 °C, respectively. Seeds incubated at 16 and 24 °C were kept for 3 months at these temperatures for germination, whereas seeds kept at 4 °C were transferred to the temperature of 16 °C after 1, 2, and 3 months to observe the effect of cold pretreatment period on germination.

2.3. Bulblet regeneration

Leaf disc, leaf scale, and bulblet explants taken from in vitro grown seedlings were used for in vitro micropropagation. Bulblets taken from in vitro grown seedlings were cut vertically into two parts, followed by culture on MS medium supplemented with 0.5–2.0 mg/L thidiazuron (TDZ). Leaf disc explants were obtained by cutting the leaf between the midrib and the edges, whereas approximately 1-cm-long leaf scale explants were taken by cutting of in vitro grown leaf into 2–3 parts. Both the leaf disc and the leaf scale explants were cultured on MS medium enriched with 2–6 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg/L kinetin. Selection of specific plant growth regulators (PGRs) for specific explants was based on the data of experiments performed on other species (data not shown).

2.4. Bulblet maturation and hardening

In vitro regenerated bulblets of different sizes (0.28–0.32 cm) were selected randomly and two different experiments were performed in order to increase the in vitro grown bulblet size for better adaptation. In the first experiment, they were cultured for 16 weeks on MS medium solidified with different concentrations and combinations of (0–6.5 g/L) agar and (0–2.25 g/L) GELRITE, devoid of PGRs. In the second experiment, bulblets were exposed for 15 days on MS solidified medium containing 10–20 g/L of NaCl, KCl, or $CaCl_2$. Data pertaining to bulblet growth and development from both experiments were scored and analyzed statistically.

2.5. Acclimatization

For acclimatization, sterile peat moss, vermiculite, sand, and perlite (2:1:1:1) were used as the substrate in all experiments. In vitro bulblets taken from the regeneration media were cultured on MS medium containing 20 g/L sucrose and placed in the refrigerator at 4 °C for a period of 2 months to break dormancy. Thereafter, these bulblets were developed further on media supplemented with different salts, transferred to trays containing compost, and incubated in growth cabinets under a 16-h light photoperiod at 23 °C and 90% humidity. The trays were covered with polyethylene bags to retain moisture inside and were opened gradually over the course of 2 weeks. Afterwards, bulblets were transferred to the greenhouse as well as to field conditions for further adaptation and growth.

2.6. Media and culture conditions

In all culture media used for in vitro germination and regeneration, the effects of solidifying agents, salts, and sucrose concentration were achieved by using MS medium supplemented with 30 g/L (w/v) sucrose, solidified with 0.65% (w/v) agar, with pH adjusted to 5.8. All media were autoclaved at 118 KPa atmospheric pressure and 120 °C for 21 min. All cultures were kept at 24 °C with a 16-h photoperiod.

2.7. Statistical analysis

In all experiments, each treatment had 3 replicates containing 10 explants depending on availability of the

material. Data taken from all experiments were subjected to statistical analysis by one-way ANOVA. SPSS 17 for Windows was used for statistical analysis and Duncan's multiple range test was employed for post hoc tests. Data presented in percentages were subjected to arcsine transformation (Snedecor and Cochran, 1967). All values were reported as means ± standard error of means.

3. Results

3.1. Seed germination

Surface sterilized seeds of *F. persica* cultured on MS medium in Magenta GA7 vessels at 4, 16, and 24 °C showed a varied response (Table 1). Seeds placed directly at 24 °C turned necrotic within 1 week and failed to germinate. Seeds at 16 °C did not show any sign of necrosis but also failed to germinate even after 3 months of culture. Seeds that were incubated at 4 °C and transferred to 16 °C after 1 month of culture also showed no germination. However, the seeds that were kept at 4 °C and transferred to 16 °C after 1 or 2 months of culture resulted in 86.7% and 96.7% germination within 2 weeks, respectively (Table 1). Furthermore, these germinated seedlings also developed green foliage and bulblets after 3 months (Figure 1a).

3.2. Bulblet regeneration

All TDZ concentrations responded well and proliferated callus induction on vertically cut half bulblet explants that was followed by shoot buds after 3 weeks of culture. However, it took a prolonged time to convert these shoots into bulblets (Figure 1b). Similarly, callus formation and shoot bud initiation started at the margins of the leaf disc and leaf scale explant on MS medium provided with 2–6 mg/L 2,4-D and 0.2 mg/L kinetin (Figure 1c) after 3 weeks. Shoot buds from both explants converted into well-developed bulblets with rooting and green foliage (Figure 1d). Both explants were allowed to remain in the culture medium for 3 more months in order to increase bulblet size (Figure 1e).

Data regarding frequency of bulblet regeneration and bulblets per explants on media containing TDZ or 2,4-D and kinetin were taken after 9 months of culture and analyzed statistically. The results clearly revealed the statistically significant effects ($P \leq 0.05$) of PGRs on bulblet regeneration. The frequency of bulblet regeneration and the number of bulblets per explant responded variably to TDZ concentrations (Figure 2) that ranged between 13.33% and 60.0% and between 4.16 and 22.86, respectively. Maximum frequency (60%) of bulblet regeneration, with a maximum number of 22.86 bulblets per explant, was noted on the medium supplemented with 2.0 mg/L TDZ. The frequency of bulblet regeneration (20.0%) with an average of 4.16 bulblets per explant was also achieved on a medium devoid of TDZ (Figure 2).

Frequency of bulblet regeneration ranged between 3.3% and 73.3% and between 30.0% and 100% for leaf disc and leaf scale explants, respectively (Figure 3). Maximum bulblet regeneration of 100% from leaf scale explants followed by 73.3% from leaf disc explants was scored on the MS medium with 2 mg/L 2,4-D and 0.2 mg/L kinetin. An increased concentration of 2,4-D significantly inhibited the frequency of bulblet regeneration on both explants. The number of bulblets per explant ranged between 2.0 and 16.93 and between 10.60 and 21.0 for leaf disc and leaf scale explants, respectively. Maximum bulblets from leaf disc explants (16.93) were recorded on MS medium with 4 mg/L 2,4-D and 0.2 mg/L kinetin. Bulblets per explant from leaf scale explants increased with increased concentration of 2,4-D, and a maximum of 21.0 bulblets was scored on MS medium with 6 mg/L 2,4-D and 0.2 mg/L kinetin (Figure 3).

3.3. Bulblet maturation and hardening

After successful regeneration, bulblets obtained from all explants were subjected to acclimatization. However, since the regenerated bulblet size was not large enough, they failed to acclimatize. Therefore, randomly selected regenerated bulblets of different sizes were cultured on MS medium with different concentrations and combinations of agar and GELRITE for 4 months. Agar-GELRITE combinations exerted positive effects on the number of secondary bulblets and roots per explant. Secondary bulblet regeneration was observed in all media from the basal part of the bulblets (Figure 4a), and on developed roots (Figure 4b). These secondary bulblets also converted into bulblets with fresh green foliage (Figure 4c).

The results showed statistically insignificant effects of agar-GELRITE combinations on bulblet diameter, which showed an increase of 0.63–1.00 cm (Table 2). The

Table 1. Effects of different temperatures and cold treatment periods on germination of *F. persica* seeds.

Germination temperature (°C)	Incubation periodat 4 °C[1]	Germination rate (%)
4	1	0.0 ± 0.00b[2]
4	2	86.7 ± 6.67a
4	3	96.7 ± 3.33a
16	-	0.0 ± 0.00b
16	-	0.0 ± 0.00b

Each value is the mean ± SE of 3 replications with 10 explants each.

[1] Seeds incubated at 4 °C were transferred to 16 °C after 1, 2, and 3 months of cold pretreatment for germination.

[2] Values in the column followed by different letters are significantly different at the 0.01 level.

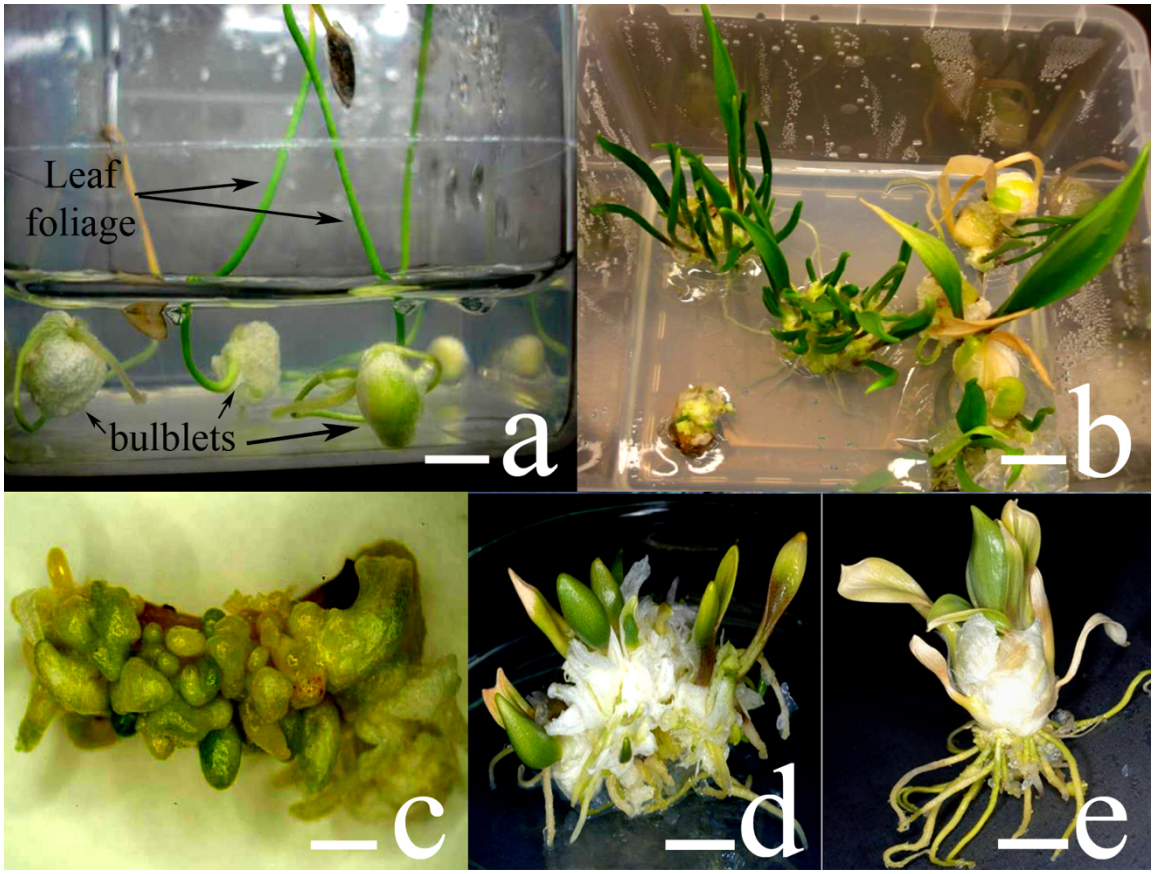

Figure 1. In vitro germination and bulblet regeneration of Persian lily. (a) In vitro germination and bulblet development on MS medium after 3 months, (b) in vitro shoot and bulblet induction from half bulblet explant cultured on MS medium with 2.0 mg/L TDZ after 6 months, (c) multiple shoot bud induction from leaf scale explant on MS medium with 2.0 mg/L 2,4-D and 0.2 mg/L kinetin after 1 month, (d) multiple shoot and bulblet induction after 3 months, and (e) well-developed bulblet after 6 months of culture (bar: a and d = 0.5 cm; b, c, and e = 1 cm).

maximum final bulblet size increase (1.00 cm) was observed on the medium solidified with a 1.5 g/L agar and 1.6 g/L GELRITE combination. Contrarily, the minimum final bulblet size increase (0.63 cm) was scored on a medium containing 5.5 g/L agar and 0.3 g/L GELRITE. The results of frequency of secondary bulblets regeneration were also insignificant and ranged between 83.3% and 100%. The minimum frequency of secondary bulblet regeneration was recorded on MS medium having 5.5 g/L agar and 0.3 g/L GELRITE. The number of secondary bulblets was statistically significant ($P \leq 0.05$), with a maximum of 4.17 secondary bulblets per explant generated on the medium supplemented with 5.5 g/L agar and 0.3 g/L GELRITE. Contrarily, the minimum number of secondary bulblets (0.33) was scored on the medium with 0.5 g/L agar and 1.92 g/L GELRITE.

Agar-GELRITE combinations exerted statistically significant effects ($P \leq 0.05$) on root length that ranged

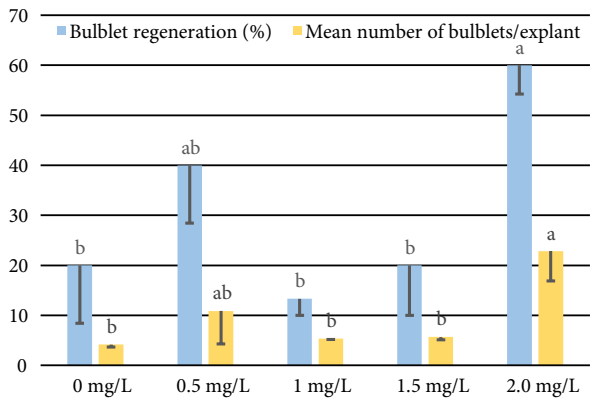

Figure 2. Bulblet regeneration from divided bulblets developed from in vitro grown seedlings of *F. persica* after 9 months in a culture on MS medium supplemented with different concentrations of TDZ. Each value is the mean ± SE of 3 replications with 10 explants each. Values followed by different letters are significantly different at the 0.05 level.

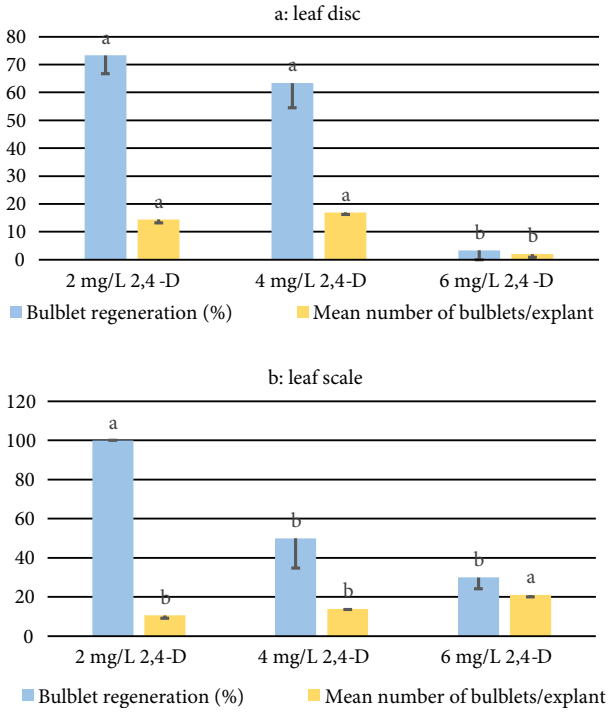

Figure 3. Bulblet regeneration from leaf disc and leaf scale explants of in vitro grown seedlings of *F. persica* after 6 months in a culture on MS medium supplemented with different concentrations of 2,4-D and 0.2 mg/L kinetin. Each value is the mean ± SE of 3 replications with 10 explants each. Values followed by different letters are significantly different at the 0.01 level.

between 2.15 and 4.88 cm (Table 2). Contrarily, the results showed statistically insignificant effects of agar-GELRITE combinations on the number of roots per bulblet that ranged between 10.67 and 16.62. The maximum number of roots per bulblet was induced on MS medium having 6.5 g/L agar only. The minimum number of roots per bulblet (10.67), with a minimum root length of 2.15 cm, was scored on the medium containing 2.25 g/L GELRITE. In general, medium solidified with 1.5 g/L agar and 1.6 g/L GELRITE was found to be the most efficient for increasing the bulblet size with the maximum change in diameter, number of roots per bulblet, and root length (Table 2).

Regenerated bulblets were also incubated on MS medium with different concentrations of NaCl, KCl, and $CaCl_2$ for 15 days in order to check their effects on bulblet growth and development. After 15 days of culture, bulblets were transferred to MS medium without salts for 4 weeks and scored. New foliage development followed by root development (Figure 4d) was observed in this period. Besides that, secondary bulblet regeneration was also obtained (Figure 4e) on the basal part of the explant. Salt type and concentration exerted statistically significant effects on rooting frequency ($P \leq 0.01$), root length ($P \leq 0.05$), number of roots ($P \leq 0.01$), and secondary bulblet regeneration ($P \leq 0.05$). Contrarily, statistically insignificant effects of salt type and concentration were observed on frequency of leaf induction, number of leaves, and secondary bulblets per explant (Table 3).

Comparing salt types, NaCl exerted positive effects on bulblet growth and development compared to other salts. NaCl (10 g/L) in the culture medium induced maximum rooting frequency (100%), root length (3.66 cm), roots per bulblet (14.58), leaf induction frequency (100), number of leaves (8.0), and number of secondary bulblets (5.0) per explant. Contrarily, $CaCl_2$ (10 g/L) produced the least response as minimum roots per bulblet (6.94) and number of leaves (3.80) were scored. Moreover, 10 g/L $CaCl_2$ also failed to induce secondary bulblet regeneration. Comparing salt concentrations, the concentration of 20 g/L, irrespective of type, was detrimental for bulblet growth and development. Minimum rooting frequency of root formation (56.7%) and root length (1.38 cm) was achieved from 20 g/L KCl and NaCl respectively, and 20 g/L KCl did not induce secondary bulblet regeneration.

3.4. Acclimatization

In vitro rooted bulblets from the regeneration medium failed to acclimatize in the pots containing different substrates under greenhouse conditions. However, plants developed new foliage and roots after 1 month when cultured at 4 °C for 2 months on MS medium containing 20 g/L sucrose followed by transfer to greenhouse conditions. In this way, a reasonable number of bulblets were adapted. Contrarily, bulblets with well-developed foliage and roots taken from agar-GELRITE mediums that were transferred to trays containing substrate developed new foliage and roots when covered with bags in a growth chamber. However, plants did not survive when they were uncovered and transferred to greenhouse conditions. Well-developed bulblets taken from salt medium developed new foliage within 1 week in the growth room. The transfer of these trays to the greenhouse exerted positive effects on foliage and root growth (Figure 4f). Plants survived for a long time and flowering was recorded under field conditions.

4. Discussion

Seed germination of *F. persica* is generally very poor due to underdeveloped embryos (Baskin and Baskin, 2004; Kizil and Khawar, 2014), which leads to physiological dormancy (Baskin and Baskin, 2004). Physiological dormancy along with abiotic factors such as temperature limits the cultivation and conservation of *F. persica* and requires alternative means of propagation (Ulug et al., 2010). In vitro germination of bulbous plants provides an efficient and alternative way of breaking the dormancy in such types of plants, by exposing the seeds to different chilling or warm temperatures for certain periods of time (Hilhorst, 2011), exogenous application of GA_3 or other phytohormones (Seo

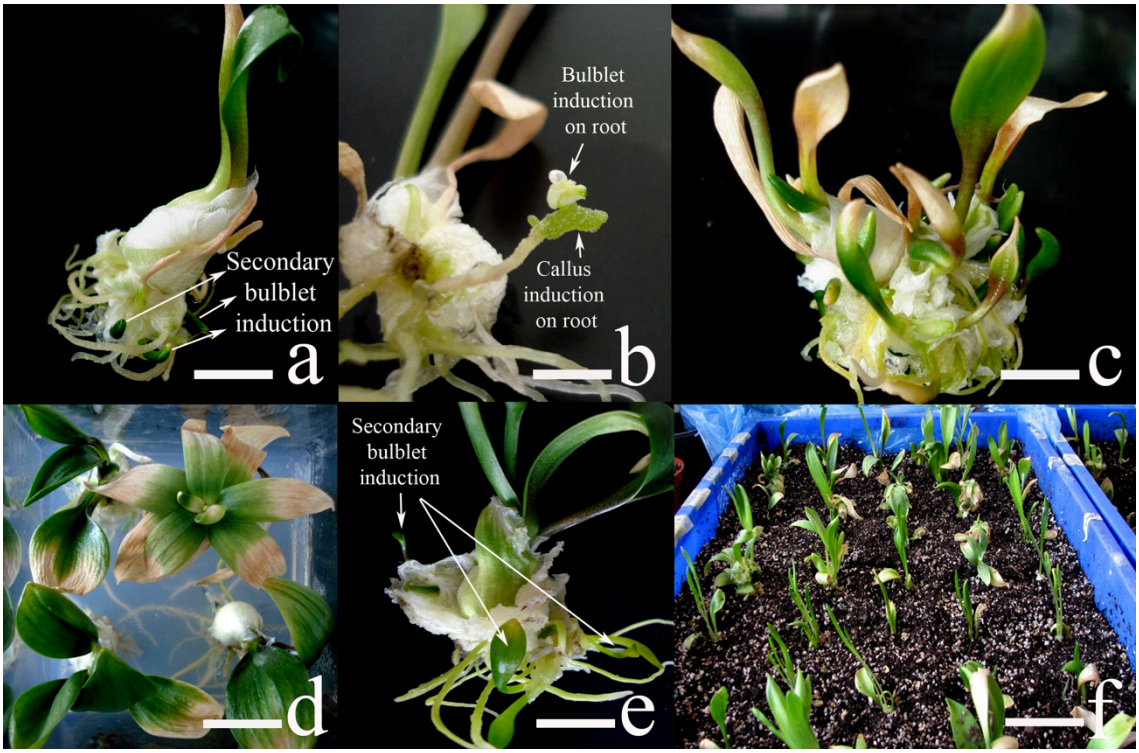

Figure 4. In vitro maturation, hardening, and acclimatization of Persian lily. (a) Secondary bulblet regeneration and (b) callus and bulblet induction on root in MS medium solidifying with agar-GELRITE. (c) Multiple bulblets induction on agar-GELRITE medium. (d) Well-established bulblets and (e) secondary bulblet induction on MS medium containing 10 g/L NaCl. (f) Acclimatized plants in trays (bar: a–e = 1 cm; f = 5 cm).

et al., 2011), light/darkness exposure or lighting source (Lian et al., 2002), or combinations of different techniques (Kizil and Khawar, 2014). The results revealed the importance of temperature on breaking the dormancy and also clearly showed the relationship between temperature and necrosis. Higher temperature led to severe necrosis, which hindered the germination process. Prolonged exposure of seeds to a low temperature (4 °C) resulted in higher germination percentage. However, prolonged exposure of germinated seedlings to a low temperature significantly hindered the seedling growth and development (Kizil and Khawar, 2014). Therefore, exposing the seedlings to a higher temperature of 16 °C ultimately proliferated the bulblets with green foliage under in vitro conditions and confirmed the recent findings of Kizil and Khawar (2014). Use of different temperature levels for the germination of *F. thunbergii* (Gao et al., 1997) and *F. tubiformis* subsp. *moggridgei* (Carasso et al., 2011) has also been reported.

Correct selection of explants for efficient regeneration is a prerequisite for bulbous plants. Explants close to meristematic zones are usually preferred for in vitro bulblet regeneration. Therefore, immature/mature embryos, bulblets, or bulbous scales explants are the first choice for in vitro bulblet regeneration of most *Fritillaria* species (Mohammadi-Dehcheshmeh et al., 2006; Özcan et al., 2007; Petric et al., 2011; Rahimi et al., 2014). Furthermore, physiological dormancy of mature or immature embryos and high contamination in bulbous plants lead to using new explants for in vitro propagation of bulbous plants. In this study, all explants showed competency to regenerate calli and bulblets using 2,4-D and kinetin, or TDZ.

Bulblet explants responded fairly well to a higher level of TDZ in the culture medium in order to gain maximum shoot regeneration. Callus induction was evident in response to high TDZ concentration, which resulted in bulblet regeneration. The results clearly revealed the relationship between TDZ concentration and regeneration response. Higher TDZ concentration boosted the bulblet regeneration frequency and bulblets per explants. Higher TDZ requirement for bulblet induction has been reported in tulip (Podwyszynska and Marasek, 2003) and *Curculigo orchiodes* (Thomas, 2007). Contrarily, Petric et al. (2011) revealed the importance of a low TDZ concentration of 0.05–0.50 mg/L for optimum bulblet formation for *F. melegris*. However, negative effects of a higher TDZ concentration (2.0 mg/L) on bulblet formation and bulblet numbers of *M. muscarimi* Medic (Uzun et al., 2014b) have also been reported.

Table 2. Effects of different agar-GELRITE concentrations and combinations in MS medium on growth and rooting of in vitro regenerated bulblets of *F. persica* after 4 months.

Agar (g/L)	GELRITE (g/L)	Increase in bulblet diameter (cm)	Frequency of secondary bulblet regeneration (%)	Mean number of secondary bulblets per explants[2]	Root length (cm)	Mean number of roots per bulblet
6.5	0	0.84 ± 0.04[ns]	100 ± 0.00[ns]	1.38 ± 1.31bc	3.56 ± 0.83abc	16.62 ± 2.45[ns]
5.5	0.3	0.63 ± 0.19	83.3 ± 8.82	4.17 ± 1.17ab	3.13 ± 0.25abc	11.88 ± 2.12
4.5	0.84	0.75 ± 0.03	100 ± 0.00	2.83 ± 1.17bc	2.66 ± 0.17c	13.66 ± 1.10
3.5	0.96	0.77 ± 0.00	100 ± 0.00	0.67 ± 0.33bc	3.03 ± 0.18bc	12.00 ± 0.95
2.5	1.28	0.73 ± 0.05	93.3 ± 6.67	3.00 ± 1.53bc	3.12 ± 0.44abc	13.97 ± 2.20
1.5	1.6	1.00 ± 0.05	100 ± 0.00	2.33 ± 0.88bc	4.50 ± 0.64ab	16.08 ± 2.14
0.5	1.92	0.91 ± 0.02	93.3 ± 6.67	0.33 ± 0.33c	4.88 ± 0.69a	12.72 ± 1.74
0	2.25	0.75 ± 0.05	100 ± 0.00	6.50 ± 1.5a	2.15 ± 0.34c	10.67 ± 1.88

Each value is the mean of 3 ± SE replications with 10 explants each.
[1] Values within a column followed by different letters are significantly different at the 0.05 level.
[2] From explants that produced bulblets.
ns: not significant.

In vitro regenerated bulblets from germination or regeneration experiments failed to acclimatize in pots containing peat moss, perlite, or vermiculite alone or in different combinations (data not shown). The main reasons are dormancy, smaller bulbous size, or maturity of in vitro grown bulblets (Langens-Gerrits et al., 2003) that did not allow for proper growth and hindered initiation of new leaves (Li and Qin, 1987) in their immediate environment. Therefore, in order to overcome these problems, a new strategy was developed using agar-GELRITE in different combinations. Solidifying agents in the culture medium alone, or in combinations, exerted significant effects on the in vitro shoot/bulblet regeneration behavior (Tsao and Reed, 2002; Uzun et al., 2014a). Agar and GELRITE are natural polysaccharides that are mostly used at certain concentrations depending mainly on explant or plant type and culture conditions (Ebrahim and Ibrahim, 2000). Our results emphasize the importance of the right combination and concentration of agar-GELRITE in the culture medium for increasing bulbous size. It is assumed that initial PGRs in the culture medium inhibited the bulblet growth, which was reversed by culturing the bulblets in a PGR-free medium with the right combination of agar and GELRITE. Positive effects of subculture or culture on PGR-free medium have been reported and credited for increasing bulblet size of other geophytes (Özel and Khawar, 2007; Özel et al., 2007; Thomas, 2007).

Besides bulblet size, secondary bulblet regeneration on the basal part of the bulblet and the root tip was of immense importance and has already been reported in other geophytes such as *Curculigo orchioides* (Thomas, 2007) and *Ornithogalum oligophyllum* (Özel and Khawar, 2007). In general, different combinations of agar-GELRITE were found to be more effective for bulblet growth and development when compared to agar or GELRITE used alone. These results are contrary to the previous findings of Ebrahim and Ibrahim (2000), who reported GELRITE to be a superior gelling agent when compared to agar alone or to combinations of agar-GELRITE for proliferation rate, organogenesis, and growth of *M. leuconeura*. Results further revealed the positive bearings of a low agar concentration in combination with GELRITE on secondary bulblet regeneration, bulblet size, change in diameter, and number of roots per bulblet and root length. It is suggested that low agar concentration increased the availability and uptake of certain nutrients like iron, calcium, and zinc (Mohamed-Yasseen, 2001; Witte et al., 2002) and lowered the concentration of toxic compounds that adversely affect plant growth (Scholten and Pierik, 1998). On the other hand, provision of GELRITE also exerted positive effects on regeneration behavior (Huang et al., 1995). Results further showed that GELRITE alone was less responsive for root induction compared to agar alone or agar and GELRITE combinations.

Salinity caused by different salts adversely affects plant growth and development by osmotic effects, specific ion toxicity, nutritional disorders, or their combinations (Grattan and Grieve, 1998; Parida and Das, 2005; Lauchli and Epstein, 2011), as well as metabolic processes, enzyme contents, and their activities (Salwa et al., 2010). These activities depend mainly on the type of species, genotypes, plant age, salinizing agent, and concentration (Jenks et al., 2007). The salts used to create salinity may adversely affect plant growth and development at a specific concentration (Munns 2002); this is a well-established phenomenon (Abou Hussien et al., 2010) under in vitro or

Table 3. Effects of different salt types and concentrations in MS medium on growth and rooting of in vitro regenerated bulblets of *F. persica*.

Salts (g/L)			Rooting (%)	Root length (cm)	Number of roots per bulblet[3]	New leaf formation (%)	Number of leaves per bulblet	Secondary bulblet regeneration (%)	Number of secondary bulblets per explant[4]
KCl	NaCl	$CaCl_2$							
10			90.0 ± 10.0ab[1]	3.44 ± 0.72a[2]	14.97 ± 1.03a[1]	93.3 ± 6.67[ns]	5.61 ± 1.43[ns]	40.0 ± 20.82a[2]	3.22 ± 2.04[ns]
20			56.7 ± 6.67c	1.83 ± 0.44b	8.00 ± 2.00b	100.0 ± 0.00	6.60 ± 1.51	0.0 ± 0.00b	0.00 ± 0.00
	10		100.0 ± 0.00a	3.66 ± 0.40 a	14.58 ± 1.97a	100.0 ± 0.00	8.00 ± 1.52	23.3 ± 3.33ab	5.00 ± 1.00
	20		73.3 ± 3.33b	1.38 ± 0.06 b	8.77 ± 0.87b	90.0 ± 10.0	5.97 ± 1.33	23.3 ± 14.53ab	3.16 ± 1.88
		10	93.3 ± 6.67ab	2.50 ± 0.29ab	6.94 ± 1.57b	93.3 ± 6.67	3.80 ± 0.76	0.0 ± 0.00b	0.00 ± 0.00

Each value is the mean ± SE of 3 replications with 10 explants each.
[1] Values within the column followed by different letters are significantly different at the 0.01 level.
[2] Values within the column followed by different letters are significantly different at the 0.05 level.
[3] From explants that produced roots.
[4] From explants that produced bulblets.
ns: not significant.

ex vitro conditions. Contrarily, our results revealed positive effects of a specific salt type and specific concentration on bulbous growth and development. Furthermore, higher concentrations, as expected, hindered growth and development. Comparing salt types, NaCl proved to be superior compared to other salts for bulbous growth as used in the study. NaCl is normally expected to have an adverse effect on plant growth and development due to suppressed cell division and restricted growth activities (Bohnert and Jensen, 1996). Accumulation of Na^+ and Cl^- in tissues led to toxicity (Tester and Davenport, 2003; Karimi et al., 2009) in the cells' cytoplasm, which affected different biochemical and physiological processes (Jampeetong and Brix, 2009). Our results revealed that bulblets tolerated a concentration of 10 g/L NaCl, which ultimately promoted the bulbous growth more efficiently as compared to 20 g/L NaCl. The positive response of bulblets to a specific salt concentration might be due to higher tolerance showed by plants at maturity, or might depend on the type of organ (Jenks et al., 2007) used in the study. Similarly, negative effects of a higher KCl concentration lead to salt stress and may affect plant growth and development by causing callus induction, necrosis, and shoot regeneration, in line with the findings of Santos et al. (2001), Sotiropoulos et al. (2006), Boyko et al. (2011), and Zahid et al. (2014). The results clearly indicate that the addition of salts at low concentrations for a specific time can be used to increase bulblet size and to harden the bulblets.

Acclimatization of in vitro regenerated bulblets is the most challenging task due to smaller size and dormancy found in the in vitro regenerated bulblets (Mirici et al., 2005; Petric et al., 2011). Therefore, optimum bulblet size with adequate rooting is a prerequisite for successful acclimatization. Researchers adopted different approaches in order to increase bulblet size prior to acclimatization. These include altering of the carbon source (Takayama and Misawa, 1979; Yaseen et al., 2013; Zhang and Jia, 2014), basal media (Naik and Nayak, 2005; Uranbey, 2010; Azad and Amin, 2013), temperature (Langens-Gerrits et al., 2003), phytohormones (Ozel et al., 2007; Aasim et al., 2008), lighting system and photoperiod (Lian et al., 2002; Aasim and Soydan, 2012; Zhang and Jia, 2014), and nitrogenous compounds (Kumar et al., 2007). Our results revealed that exposing the bulblets to 10 g/L NaCl helped the adaptation of the in vitro regenerated bulblets. Furthermore, adaptation of in vitro regenerated bulblets in a growth chamber with controlled conditions can also be employed, but only for small-scale production. Use of specific substrate (peat moss, vermiculite, sand, and perlite; 2:1:1:1) is also important and may vary with plant type (Petric et al., 2011; Rahimi et al. 2014). Results further revealed the importance of sucrose concentration on hardening and acclimatization. Application of a reduced exogenous sucrose concentration has been successfully employed for hardening and acclimatization of other *Fritillaria* species. Contrarily, higher concentrations were used for embryo maturation and induction of storage proteins, fatty acids, and others required for embryo germination (Rai et al., 2008, 2011).

In conclusion, our study presents an improved, efficient, and reliable in vitro bulblet regeneration protocol of the Persian lily (*F. persica*). In this study, we successfully established in vitro seed germination followed by induction of singlet bulblets. Thereafter, in vitro multiple bulblet regeneration was successfully established using different explants and PGRs. The study also presents a successful use of different gelling agents and salt concentrations for maturation of these bulblets. In the last step, we demonstrated a number of new acclimatization strategies for successful adaptation to their natural environment. In short, these protocols can be employed for commercial-scale propagation of *Fritillaria* or other geophytes in future studies.

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References

Aasim M, Khawar K, Özcan S (2008). In vitro regeneration of red squill *Urginea maritima* (L.) Baker. using thidiazuron. Biotechnol Biotec Eq 22: 925-928.

Aasim M, Soydan B (2012). Effect of LED lights and sucrose concentration on rooting and bulblets size of *Muscari muscarimi* Medicus. In: Proceedings of XI International Symposium on Flower Bulbs and Herbaceous Perennials Conference, p. 251.

Abou Hussien E, El-Shinnawi M, Abo El-Fadl M, El-Fishey M (2010). Growth of corn plants cultivated in differently manured arid soils and irrigated with various water qualities. Minufiya Journal of Agricultural Research 29: 335-351.

Azad MAK, Amin MN (2013). Effects of hormonal and basal nutrient medium on in vitro regeneration of an ornamental plant – *Muscari armeniacum* Leichtlin. ex Baker. Plant Tissue Culture and Biotechnology 22: 113-126.

Baskin JM, Baskin CC (2004). A classification system for seed dormancy. Seed Sci Res 14: 1-16.

Bohnert HJ, Jensen RG (1996). Metabolic engineering for increased salt tolerance – the next step. Funct Plant Biol 23: 661-667.

Boyko A, Matsuoka A, Kovalchuk I (2011). Potassium chloride and rare earth elements improve plant growth and increase the frequency of the *Agrobacterium tumefaciens*-mediated plant transformation. Plant Cell Rep 30: 505-518.

Bryan JE (2002). Bulbs. Revised Edition. Portland, OR, USA: Timber Press.

Carasso V, Hay FR, Probert RJ, Mucciarelli M (2011). Temperature control of seed germination in *Fritillaria tubiformis* subsp. *moggridgei* (Liliaceae) a rare endemic of the South-west Alps. Seed Sci Res 21: 33-38.

Ebrahim MK, Ibrahim IA (2000). Influence of medium solidification and pH value on in vitro propagation of *Maranta leuconeura* cv. Kerchoviana. Sci Hortic-Amsterdam 86: 211-221.

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 59: 197-201.

Gao WY, Li ZL, Xiao PG (1997). Temperature effects on seed dormancy relieving of *Fritillaria thunbergii* Miq. Journal of Chinese Pharmaceutical Sciences 6: 160-164.

Grattan S, Grieve C (1998). Salinity–mineral nutrient relations in horticultural crops. Sci Hortic-Amstedam 78: 127-157.

Hilhorst HW (2011). Standardizing seed dormancy research. Methods Mol Biol 773: 43-52.

Huang LC, Kohashi C, Vangundy R, Murashige T (1995). Effects of common components on hardness of culture media prepared with Gelrite™. In Vitro Cell Dev-Pl 31: 84-89.

Jampeetong A, Brix H (2009). Effects of NaCl salinity on growth, morphology, photosynthesis and proline accumulation of *Salvinia natans*. Aquat Bot 91: 181-186.

Jenks MA, Hasegawa PM, Jain SM, Foolad M (2007). Advances in molecular breeding toward drought and salt tolerant crops. Amsterdam, the Netherlands: Springer.

Karimi E, Abdolzadeh A, Sadeghipour HR (2009). Increasing salt tolerance in Olive, *Olea europaea* L. plants by supplemental potassium nutrition involves changes in ion accumulation and anatomical attributes. Int J Plant Prod 3: 49-60.

Kizil S, Khawar KM (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.

Kukulczanka K, Kromer K, Czastka B (1988). Propagation of *Fritillaria meleagris* L. through tissue culture. In: Proceedings of III International Symposium on Growth Regulators in Ornamental Horticulture Conference, pp. 147-154.

Kumar S, Awasthi V, Kanwar, J (2007). Influence of growth regulators and nitrogenous compounds on in vitro bulblet formation and growth in oriental lily. Hort Sci (Prague) 34: 77-83.

Langens-Gerrits M, De Klerk GJ, Croes A (2003). Phase change in lily bulblets regenerated in vitro. Physiol Plantarum 119: 590-597.

Lauchli A, Epstein E (2011). Plant responses to saline and sodic conditions. In: Wallender WW, Tanji KK, editors. Agricultural Salinity Assessment and Management. 2nd ed. Reston, VA, USA: American Society of Civil Engineers, pp. 169-205.

Le Nard M, De Hertogh A (1993). Plant breeding and genetics. In: De Hertogh AA, Le Nard M, editors. The Physiology of Flower Bulbs: A Comprehensive Treatise on the Physiology and Utilization of Ornamental Flowering Bulbous and Tuberous Plants. Amsterdam, the Netherlands: Elsevier, pp. 161-169.

Li C, Qin Z (1987). The relationship between physiological and biochemical changes and releasing dormancy of *Fritillaria pallidiflora* Schrenk. during low temperature treatment. Acta Botanica Boreali-Occidential Sinica 7: 23-28.

Li SL, Lin G, Chan SW, Li P (2001). Determination of the major isosteroidal alkaloids in bulbs of *Fritillaria* by high-performance liquid chromatography coupled with evaporative light scattering detection. J Chromatogr A 909: 207-214.

Lian ML, Murthy H, Paek KY (2002). Effects of light emitting diodes (LEDs) on the in vitro induction and growth of bulblets of *Lilium* oriental hybrid 'Pesaro'. Sci Hortic-Amsterdam 94: 365-370.

Mirici S, Parmaksız I, Özcan S, Sancak C, Uranbey S, Sarıhan EO, Gümüşcü A, Gürbüz B, Arslan N (2005). Efficient in vitro bulblet regeneration from immature embryos of endangered *Sternbergia fischeriana*. Plant Cell Tiss Org 80: 239-246.

Mohamed-Yasseen Y (2001). Influence of agar and activated charcoal on uptake of gibberellin and plant morphogenesis in vitro. In Vitro Cell Dev-Pl 37: 204-205.

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*. HortScience 41: 1066-1066.

Munns R (2002). Comparative physiology of salt and water stress. Plant Cell Environ 25: 239-250.

Naik PK, Nayak S (2005). Different modes of plant regeneration and factors affecting in vitro bulblet production in *Ornithogalum virens*. ScienceAsia 31: 409-414.

Özcan S, Parmaksiz I, Mirici S, Çöçü S, Uranbey S, Ipek A, Sancak C, Sarihan EO, Gürbüz B, Sevimay CS (2007). Efficient in vitro bulblet regeneration from immature embryos of endemic and endangered geophyte species in *Sternbergia*, *Muscari*, and *Fritillaria* genera. In: Biotechnology and Sustainable Agriculture 2006 and Beyond. Conference Proceedings. Berlin, Germany: Springer, pp. 381-383.

Ozel CA, Khawar KM (2007). In vitro bulblet regeneration of *Ornithogalum oligophyllum* E. D. Clarke using twin scale bulb explants. Propag Ornam Plants 7: 82-88.

Ozel CA, Khawar KM, Unal F (2007). In vitro axillary bulblet regeneration of Turkish Yellow Grape Hyacinth (*Muscari macrocarpum* Sweet) from twin scale explants. Research Journal of Agriculture and Biological Sciences 3: 924-929.

Paek K, Murthy H (2002). High frequency of bulblet regeneration from bulb scale sections of *Fritillaria thunbergii*. Plant Cell Tiss Org 68: 247-252.

Paek K, Sung N, Park C (1996). Several factors affecting bulblet regeneration from the culture of scale segment and node-bud in fritillary as medicinal bulbous plant. In: Proceedings of International Symposium on Plant Production in Closed Ecosystems Conference, pp. 498-503.

Parida AK, Das AB (2005). Salt tolerance and salinity effects on plants: a review. Ecotox Environ Safe 60: 324-349.

Petric M, Subotic A, Jevremovic S, Milana Trifunovic-Momcilov M (2011). Somatic embryogenesis and bulblet regeneration in snakehead fritillary (*Fritillaria meleagris* L.). Afr J Biotechnol 10: 16181-16188.

Podwyszynska M, Marasek A (2003). Effects of thidiazuron and paclobutrazol on regeneration potential of tulip flower stalk explants in vitro and subsequent shoot multiplication. Acta Soc Bot Pol 72: 181-190.

Rahimi M, Daneshvar MH, Heidari M, Ahvaz I (2014). Propagation and bulb formation of Fritillaria (*Fritillaria imperialis*) via in vitro culture. International Journal of Plant Animal and Environmental Sciences 4: 707-710.

Rai MK, Jaiswal V, Jaiswal U (2008). Effect of ABA and sucrose on germination of encapsulated somatic embryos of guava (*Psidium guajava* L.). Sci Hortic-Amsterdam 117: 302-305.

Rai MK, Shekhawat N, Gupta AK, Phulwaria M, Ram K, Jaiswal U (2011). The role of abscisic acid in plant tissue culture: a review of recent progress. Plant Cell Tiss Org 106: 179-190.

Rønsted N, Law S, Thornton H, Fay MF, Chase MW (2005). Molecular phylogenetic evidence for the monophyly of *Fritillaria* and *Lilium* (Liliaceae; Liliales) and the infrageneric classification of *Fritillaria*. Mol Phylogenet Evol 35: 509-527.

Salwa A, Hammad KA, Tantawy M (2010). Studies on salinity tolerance of two peanut cultivars in relation to growth, leaf water content. Some chemical aspects and yield. Journal of Applied Sciences Research 6: 1517-1526.

Santos CV, Campos A, Azevedo H, Caldeira G (2001). In situ and in vitro senescence induced by KCl stress: nutritional imbalance, lipid peroxidation, and antioxidant metabolism. J Exp Bot 52: 351-360.

Scholten H, Pierik R (1998). Agar as a gelling agent: chemical and physical analysis. Plant Cell Rep 17: 230-235.

Seo M, Jikumaru Y, Kamiya Y (2011). Profiling of hormones and related metabolites in seed dormancy and germination studies. In: Kermode AR, editor. Seed Dormancy Methods and Protocols. New York, NY, USA: Humana Press, pp. 99-111.

Snedecor GW, Cochran WG (1967). Statistical Methods. Ames, IA, USA: The Iowa State University Press, pp. 327-329.

Sotiropoulos T, Dimassi K, Tsirakoglou V, Therios I (2006). Responses of two *Prunus* rootstocks to KCl induced salinity in vitro. Biol Plantarum 50: 477-480.

Sun C, Chu C, Wang C (1977). Callus formation and organ regeneration in the tissue culture of *Fritillaria thunbergii* Miq. Acta Bot Sin 19: 161-162.

Takayama S, Misawa M (1979). Differentiation in *Lilium* bulbscales grown in vitro. Effect of various cultural conditions. Physiol Plantarum 46: 184-190.

Tester M, Davenport R (2003). Na^+ tolerance and Na^+ transport in higher plants. Ann Bot-London 91: 503-527.

Thomas TD (2007). High-frequency, direct bulblet induction from rhizome explants of *Curculigo orchioides* Gaertn., an endangered medicinal herb. In Vitro Cell Dev-Pl 43: 442-448.

Tsao C, Reed B (2002). Gelling agents, silver nitrate and sequestrene iron influence adventitious shoot and callus formation from Rubus leaves. In Vitro Cell Dev-Pl 38: 29-32.

Ulug BV, Korkut AB, Sisman EE, Ozyavuz M (2010). Research on propagation methods of Persian lily Bulbs [*Fritillaria persica* Linn.] with various vegetative techniques. Pak J Bot 42: 2785-2792.

Uranbey S (2010). Stimulating effects of different basal media and cytokinine types on regeneration of endemic and endangered *Muscari aucheri*. Arch Biol Sci 62: 663-667.

Uzun S, İlbaş Aİ, İpek A, Arslan N, Barpete S (2014a). Efficient in vitro plant regeneration from immature embryos of endemic *Iris sari* and *I. schachtii*. Turk J Agric For 38: 348-353.

Uzun S, Parmaksız İ, Uranbey S, Mirici S, Sarıhan EO, İpek A, Kaya MD, Gürbüz B, Arslan N, Sancak C et al. (2014b). In vitro micropropagation from immature embryos of the endemic and endangered *Muscari muscarimi* Medik. Turk J Biol 38: 83-88.

Wang S, Gao W, Chen H, Xiao P (2005). New starches from *Fritillaria* species medicinal plants. Carbohyd Polym 61: 111-114.

Witte CP, Tiller SA, Taylor MA, Davies HV (2002). Addition of nickel to Murashige and Skoog medium in plant tissue culture activates urease and may reduce metabolic stress. Plant Cell Tiss Org 68: 103-104.

Yaseen M, Ahmad T, Sablok G, Standardi A, Hafiz IA (2013). Review: role of carbon sources for in vitro plant growth and development. Mol Biol Rep 40: 2837-2849.

Zahid MN, Hasan M, Adil M, Hossain MM, Mian MAK (2014). In vitro screening for salt tolerance in aromatic rice genotypes. Open Science Journal of Bioscience and Bioengineering 1: 28-32.

Zhang M, Jia G (2014). The effects of sucrose concentration and light condition on lily's bulblet-in-tube production and inclusion content. Pak J Bot 46: 307-315.