Research Article

Somatic embryogenesis and encapsulation of immature bulblets of an ornamental species, grape hyacinths (*Muscari armeniacum* Leichtlin ex Baker)

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Abstract: An efficient in vitro regeneration system was developed for the ornamental *Muscari armeniacum* on Linsmaier and Skoog basal medium (LS) supplemented with benzyladenine (BA) at varying concentrations (0.5, 1.0, or 2.0 mg/L) alone or in combination with 0.5 mg/L α-naphthalene acetic acid (NAA). The highest mean number of direct somatic embryo formations was observed on LS medium containing 2.0 mg/L BA and 0.5 mg/L NAA, with a mean of 7.9 somatic embryos per explant after 10 weeks of culture. Green nodular calli induced on LS medium containing 5.0 mg/L BA alone or in combination with 0.5 mg/L NAA were transferred to LS medium supplemented with or without 0.5 mg/L gibberellic acid (GA3) for 8 weeks, producing 23.3 immature bulblets. Immature bulblets produced in vitro were either embedded in a sodium alginate matrix for the encapsulation process or were transferred directly to LS medium supplemented with or without GA_3 at 0.5 or 1.0 mg/L for growth and development for 6 weeks. Encapsulated bulblets were then stored at 4 °C in darkness for 10 weeks and almost all encapsulated bulblets retained their viability and resumed their growth under nonaxenic greenhouse conditions.

Key words: Encapsulation, *Muscari armeniacum*, ornamental plants, somatic embryogenesis

1. Introduction

The genus *Muscari* has 47 species according to the World Checklist of Selected Plant Families (http://apps.kew.org/wcsp) as of May 2013; only 4 or 5 are widely available for horticultural practices and can be found commonly in markets. In the Turkish flora, there are 29 *Muscari* species, and most of them are listed as endemic and endangered (Eker and Koyuncu, 2008). *Muscari armeniacum* is commonly known as grape hyacinth owing to its clusters of small, bell-shaped, cobalt-blue flowers that look like clusters of upside-down grapes (Grey-Wilson et al., 1981). *M. armeniacum*, belonging to the family Asparagaceae, is cultivated in pots and gardens in the temperate regions. Traditional methods of propagation of *Muscari* species are rather slow, since the bulblet production from the mother bulbs is extremely low (Uzun et al., 2014). Tissue culture applications might be appropriate for the bulblet production in vitro and might also be assessed as a prevailing instrument for ex situ conservation of geophytes against their intensive collection from natural habitats. However, micropropagation of several *Muscari* species, such as *M. racemosum* (Kromer, 1989), *M. macrocarpum* Sweet (Ozel et al., 2007), *M. aucheri* (Uranbey, 2010a),

M. azureum (Uranbey, 2010b), *M. mirium* (Nasırcılar et al., 2011), and *M. muscarimi* (Uzun et al. 2014), has been established. There are also some reports concerning plantlet regeneration of *M. armeniacum* via callus cultures (Suzuki and Nakano, 2001; Mori and Nakano, 2004; Azad and Amin, 2012) and protoplast cultures (Nakano et al., 2005).

Encapsulation technology provides easy handling of living materials; new understanding towards the storability, uniformity, and the use of sowing equipment; and a barrier against the transmission of pathogens (Patel et al., 2000; Winkelmann et al., 2003). This study describes a simple and effective protocol for both in vitro direct and indirect somatic embryo production from scale explants for subsequent bulblet formation followed by encapsulation of immature bulblets for conservation and storage purposes without disturbing the natural habitats for the first time in an ornamental species.

2. Materials and methods

2.1. Plant materials and embryo induction
Mature bulbs of *Muscari armeniacum* collected from the Gölköy Campus of Abant İzzet Baysal University were

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stored at 4 °C for 1 week prior to explant excision. Surface sterilization of the bulbs was carried out by washing the bulbs thoroughly under running tap water followed by surface sterilization with a 20% (v/v) commercial bleach solution [Domestos; approximately 5% (v/v) sodium hypochlorite] for 20 min, and were finally rinsed 4 times with sterile distilled water. The outer bulb scales were removed, and the innermost scales were used as the explant source (Figure 1a). Explants were cut into 2 or 3 pieces vertically (ca. 0.5 cm) and were then placed with their abaxial surfaces in contact with Linsmaier and Skoog (LS) medium (Linsmaier and Skoog, 1965) containing 3% (w/v) sucrose, 0.8% (w/v) plant agar, and different concentrations of benzyladenine (BA) and naphthalene acetic acid (NAA; Table 1). The pH of the medium was adjusted to 5.7 before the addition of agar and it was autoclaved at 121 °C for 20 min. Five explants in triplicate were used for each plastic petri dish (90 × 10 mm) containing approximately 25 mL of medium. Data on the percentage of explants performing embryogenesis, mean number of somatic embryos, and bulblet formation were recorded after 6 and 10 weeks of culture, respectively.

Following 10 weeks of culture, green nodular embryonic calli that separated from the scale explants (mainly in an adaxial position in contact with medium) were transferred to LS medium with or without 0.5 mg/L gibberellic acid (GA$_3$) for embryo induction for 8 weeks (Table 2). Five callus clumps (approximately 100 mg each) in triplicate were used (i.e. a total of 15 callus clumps per treatment) and were subcultured every 3 weeks. Data on globular and cotyledonary embryo formation on callus tissue were recorded after 6 and 8 weeks of culture, respectively.

2.2. Plant growth and development

In vitro produced bulblets obtained via direct somatic embryogenesis were placed on culture tubes containing 15 mL of LS medium supplemented with 1.5% sucrose (w/v) and 0.8% (w/v) plant agar with or without GA$_3$ at 2 different concentrations (0.5 or 1.0 mg/L). Shoot and root sizes were recorded after 6 weeks of cultivation. All cultures were incubated in a growth chamber at 23 ± 1 °C with relative humidity of about 55% under humid conditions with a 16-h photoperiod (approximately 35 µmol m^{-2} s^{-1}).

2.3. Encapsulation of the bulblets

Encapsulation in conventional alginate beads was carried out using the slightly modified protocol of Winkelmann et al. (2004). Immature bulblets produced in vitro (approximately 0.5 cm in diameter at the cotyledonary stage produced from green nodular callus clumps), along with 150 µL of a sodium alginate (1.5% w/v) solution, were individually removed using a micropipette (1000 µL) with a cut tip to provide a wider opening. Each droplet containing 1 randomly selected immature bulblet was dropped into a calcium chloride solution (1% w/v) in a 250-

mL flask. Solution mix and gel beads were stirred at 120 rpm in an orbital shaker to induce further polymerization. After several washes of all alginate beads with distilled water, they were collected on a metal sieve and placed into centrifuge tubes (2 mL in size). Encapsulated bulblets were stored at 4 °C in a refrigerator for 10 weeks. All solutions used during the encapsulation process were autoclaved. All chemical compounds used in tissue culture experiments were purchased from Duchefa (the Netherlands).

2.4. Hardening off

After carefully removing agar residue with tap water, regenerated plantlets with well-developed bulblets with roots were transferred to pots (Gardman 40 cell insert, UK) containing compost (pH 6.2, nitrogen 0.12%, Stender, Germany). Sowing of the encapsulated bulblets on compost was done after 10 weeks of storage in a refrigerator at 4 °C. The pots were watered 3 times a day for 2 weeks. After leaves emerged, water was sprayed twice a day.

2.5. Data analysis

Somatic embryo number, bulblet number, and shoot and root sizes were presented as mean ± standard error. The mean number of direct somatic embryo formations and frequency of explants forming embryos were calculated, with plant growth regulator (PGR) concentrations as the independent variable. The same method was applied for the measurements of shoot and root size. An analysis of variance test (one-way) with Duncan's test was used to estimate differences between treatment means at P = 0.05 using STATISTICA version 8.0.

3. Results

3.1. Bulblet production from somatic embryos

Somatic embryo induction and the rate of bulblet formation from scale explants were dependent on concentrations of BA alone or in combination with NAA (Table 1). Direct somatic embryos were clearly observed at the adaxial surface of the explants after 6 weeks of cultivation. Higher mean numbers of direct somatic embryos (Figure 1b) and bulblet formations (Figure 1c) were obtained when the LS culture medium contained a combination of 2.0 mg/L BA and 0.5 mg/L NAA; this treatment resulted in 7.9 somatic embryos per explant and 39.7 bulblets per treatment. It was followed by 6.4 somatic embryos per explant and 32.0 bulblets per treatment on LS medium containing 1.0 mg/L BA and 0.5 mg/L NAA. On the other hand, 2.0 mg/L BA alone induced 6.6 somatic embryos per explant and 33.0 bulblets per treatment, and the mean number of somatic embryos drastically decreased with 1.0 mg/L BA used alone. There was a significant difference (P < 0.05) between the treatments using 1.0 mg/L BA alone and its combination with 0.5 mg/L NAA (2.5 and 6.4 somatic embryos per explant; Table 1). While BA alone at its lowest concentration (0.5 mg/L) was not effective for embryo

Table 1. Effects of different concentrations of BA alone or in combination with 0.5 mg/L NAA on the development of direct somatic embryos (after 6 weeks of culture) and bulblets (after 10 weeks of culture)

Treatments	Mean number of somatic embryos per scale explant[x]	% of explants producing direct somatic embryos	Mean number of bulblets per treatment[y]	Description of callus response
No PGRs	0[e]	0	0[e]	No callus
0.5 BA	0[e]	0	0[e]	Hard, yellow to green amorphous callus at abaxial surface
1.0 BA	2.5 ± 0.4[d]	87	12.3 ± 3.8[d]	No callus
2.0 BA	6.6 ± 1.2[ab]	87	33.0 ± 9.3[ab]	Light-yellow to cream-colored nodular callus at abaxial surface
5.0 BA	0[e]	0	0[e]	A massive white to light-yellow friable callus
0.5 BA + 0.5 NAA	3.5 ± 0.7[c]	87	17.7 ± 2.9[c]	White to light-yellow friable callus at abaxial surface
1.0 BA + 0.5 NAA	6.4 ± 0.6[b]	100	32.0 ± 4.3[ab]	Light-yellow to cream-colored nodular callus at abaxial surface
2.0 BA + 0.5 NAA	7.9 ± 0.3[a]	100	39.7 ± 1.7[a]	No callus
5.0 BA + 0.5 NAA	0[e]	0	0[e]	Light-yellow to green-colored nodular callus

.[x]: Means with the same letter within a column are not significantly different at a 95% confidence interval, ± represents standard error of the mean values.
Data collected after 6[x] and 10[y] weeks of culture.

production, it was found to be moderately effective when combined with 0.5 mg/L NAA, producing 3.5 somatic embryos per explant. After 10 weeks of incubation, neither the control group nor treatments containing 5.0 mg/L BA alone or in combination with 0.5 mg/L NAA produced direct somatic embryos on scale explants (Table 1).

In addition to direct somatic embryogenesis, green nodular calli from light-yellow protuberances on the entire abaxial surface of scale explants on LS medium containing 5.0 mg/L BA alone or in combination with 0.5 mg/L NAA (Table 1; Figure 1d) were used for indirect somatic embryogenesis and transferred onto LS medium with or without 0.5 mg/L GA_3 (Table 2). Globular embryo formation was clearly observed in high numbers on LS basal medium (28.9 embryos per callus) after 6 weeks (Table 2; Figure 1e). There was no difference between LS basal medium or LS containing 0.5 mg/L GA_3 in terms of the mean number of globular embryos per callus clump. At the end of 8 weeks of cultivation, a large number of globular embryos developed into the cotyledonary stage, producing 23.3 or 20.8 embryos per callus clump on LS basal medium or LS supplemented with 0.5 mg/L GA_3, respectively (Table 2; Figure 1f).

3.2. Growth, development, encapsulation, and hardening off

Further maintenance of the cultures for up to 6 weeks, i.e. the 14th week of cultivation, resulted in a continuous development of roots and shoots from bulblets (Figure 1g) cultured on LS medium with or without GA_3 (0.5 or 1.0 mg/L; Figure 2). There was no significant difference (P < 0.05) between different concentrations of GA_3 tested (0.5 or 1.0 mg/L). Plantlet development was successful when the immature bulblets were cultured on LS medium supplemented with GA_3 (i.e. an average of 3.3 cm leaf and 2.4 cm root size at 0.5 mg/L GA_3). LS medium without PGRs resulted in a weak response for the growth and development of the bulblets (Figure 2). The plantlets derived from in vitro cultures were transferred to the pots containing compost. Nearly all of the plants (95%) survived through the hardening off process (Figure 1h). Furthermore, phenotypic variation was not observed in the regenerated plantlets.

For encapsulation, well-developed immature bulblets derived from green nodular embryonic calli (Table 2) were encapsulated (Figure 3a) within a sodium-alginate (1.5% w/v) matrix. Each bulblet was embedded into a bead
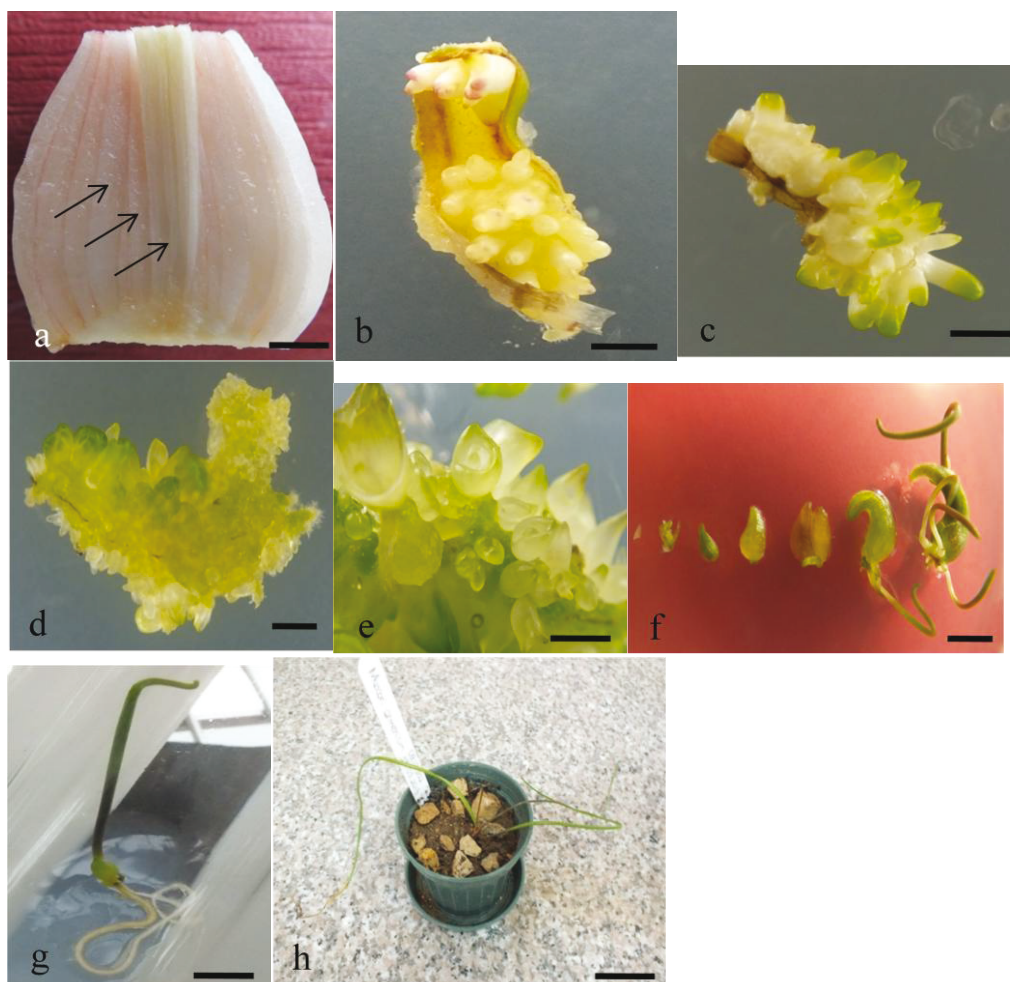
Figure 1. In vitro bulblet regeneration, rooting, and acclimatization of *Muscari armeniacum*: (a) innermost scales shown by arrows as explant source, (b) somatic embryo formation after 6 weeks or (c) after 10 weeks of cultivation on LS medium containing 2.0 mg/L BA, (d) callus clumps on LS medium with or without 0.5 mg/L GA$_3$, (e) multiple somatic embryo formation within 6 weeks, (f) an overview of the somatic embryogenesis, (g) a well-rooted and sprouted bulblet on LS medium supplemented with 0.5 mg/L GA$_3$ for 6 weeks, (h) hardening off process under greenhouse conditions. Scale bar: a = 5.0 mm; b = 2.5 mm; c = 3.0 mm; d = 2.0 mm; e = 1.5 mm; f = 10 mm; g = 30 mm; h = 80 mm.

Table 2. Embryonic development of green nodular callus tissue from globular to cotyledonary stages on LS medium with or without 0.5 mg/L GA$_3$.

	Somatic embryos per callus at globular stage (after 6 weeks)	Somatic embryos per callus at cotyledonary stage (after 8 weeks)
No PGRs	28.9 ± 1.9	23.3 ± 2.1
0.5 GA$_3$	28.5 ± 1.5	20.8 ± 1.0

± represents standard error of the mean values.

(gel matrix) and then placed into the microcentrifuge tubes (i.e. 1 bead into a single 2-mL tube, closed tightly; Figure 3b). In order to minimize the desiccation and vegetative regrowth of the embryos during storage, the use of tightly closed centrifuge tubes (2 mL in volume) was found to be more successful than petri dishes (data not
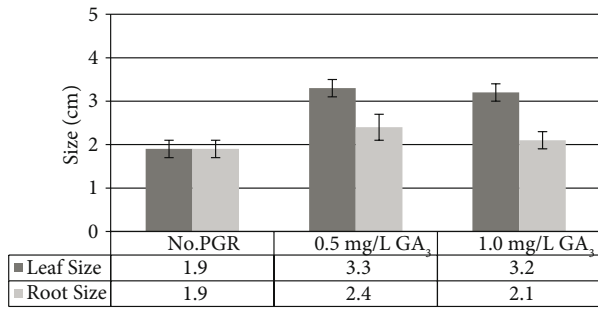
Figure 2. Shoot and root size (cm) of bulblets incubated on LS medium with GA₃ after 6 weeks of cultivation. Bars on bar graphs represent standard error of the means.

	No.PGR	0.5 mg/L GA₃	1.0 mg/L GA₃
■ Leaf Size	1.9	3.3	3.2
▫ Root Size	1.9	2.4	2.1

shown). Each tube was stored at 4 °C in darkness for 10 weeks. Afterwards, gel beads were placed into fingertip-sized holes created on the surface of compost (Figure 3c). Almost all encapsulated bulblets sprouted within 2 weeks after planting them directly in the compost. The soil was fully watered every day with tap water for the first 2 weeks. Thereafter, when plume sheath was clearly visible (ca. 1.0 or 1.5 cm), spraying the plantlets with water 2 or 3 times a day was found to be very effective for further growth and development (data not shown). A great majority of the

encapsulated bulblets (13 out of 15 encapsulated bulblets) sprouted under nonaxenic greenhouse conditions with fully expanded leaves (ca. 8 cm in size) within 6 weeks (Figures 3d–3f).

4. Discussion

In vitro propagation of *M. armeniacum* was successfully established, producing either direct somatic embryogenesis or embryonic callus cultures. There have been several reports in which different types of explants and medium formulations were tested in several *Muscari* species. Nasırcılar et al. (2011) compared 2- and 4-scale explants, and they obtained the highest bulblet formation in *M. mirium* on MS medium supplemented with 4.0 mg/L BA and 0.25 mg/L NAA. Uranbey (2010a, 2010b) reported in vitro bulblet formation from bulb scales of *M. azureum* and *M. aucheri* with different medium formulations. Similarly, there are some reports concerning somatic embryogenesis in bulb explants of *M. neglectum* inoculated with various concentrations of BA alone or in combination with NAA (Karamian et al., 2010) and encapsulation of somatic embryos obtained from protoplast cultures (Karamian et al., 2011). Recently, Uzun et al. (2014) reported the best bulblet regeneration
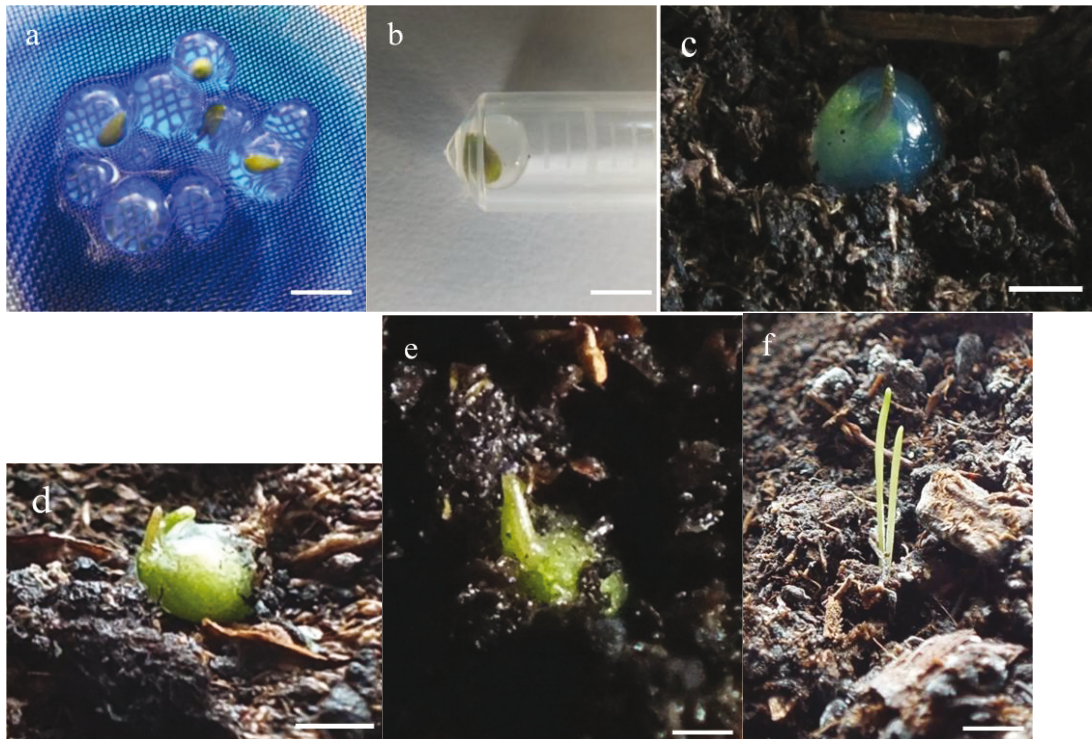
Figure 3. Encapsulation and germination of in vitro regenerated bulblets: (a) sodium alginate encapsulation of the bulblets produced in vitro, (b) storage of the bulblets in gel beads in 2-mL centrifuge tubes for 10 weeks in a fridge at 4 °C, (c) sowing the gel beads on compost, (d) growth of plumule leaves after 1, (e) 2, and (f) 6 weeks. Scale bars: a, b, e, f = 10 mm; c, d = 15 mm.

in *M. muscarimi* using MS medium containing 4.0 mg/L BA and 0.50 mg/L NAA after 1 year of culture initiation. On the other hand, there have been several reports on tissue culture studies via leaf and callus cultures derived from flower buds (Suzuki and Nakano, 2001; Mori and Nakano, 2004; Azad and Amin, 2012), protoplast cultures of the *Muscari* cultivar Blue Pearls (Nakano et al., 2005), and *Agrobacterium*-mediated production of transgenic *M. armeniacum* (Suzuki and Nakano, 2002). Even though we used comparable concentrations and combinations of BA and NAA as reported earlier for the regeneration of several *Muscari* species, including *M. armeniacum*, our findings were not consistent with these reports in terms of callus induction patterns. Suzuki and Nakano (2001) reported callus induction pattern for organogenesis and embryogenesis using leaf explants and found a low rate of callus induction in bulb scale explants. However, our data showed that bulb scale explants were effective for both direct and indirect somatic embryogenesis, depending on medium formulations. In contrast to our findings, Suzuki and Nakano (2001) and Mori and Nakano (2004) reported that bulb scale explants were not responsive for organogenic callus induction. These differences could be due to the distinct geographical origin of the species as well as varying physiological responses of natural populations and their cultivar plants to the regeneration media used.

The innermost scale explants excised from surface-sterilized bulbs are very effective explant sources in the family Liliaceae (Liu and Yang, 2012). In our study, bulb scale explant positions in culture media and the concentration of PGR combinations played a critical role for the regeneration and development in vitro. Furthermore, the epidermal layer on the abaxial surface being in contact with the medium might have increased embryonic callus formation at high dosages of BA (2.0 or 5.0 mg/L). This finding was also consistent with an earlier report in which the quantification of cell proliferation associated with veins in both adaxially and abaxially plated explants reinforced the large amount of cell proliferation associated with the vascular tissue when explants are plated abaxial-side down (Wang et al. 2011). In our study, light-yellow to green nodular protuberances were the general responses of callus tissue that developed within 10 weeks of cultivation (Table 1). Two types of tissue culture system were developed in our study: direct and indirect somatic embryogenesis via green nodular callus. In spite of direct somatic embryogenesis, a higher level of somatic embryo formation was achieved via a callus-mediated tissue culture system. Therefore, handling of callus tissue as a stock culture was found to be much more efficient than scale explants excised from mature bulblets for plant propagation. When bulblets were incubated on

LS medium supplemented with or without GA$_3$ (0.5 or 1.0 mg/L), either concentration of GA$_3$ was more effective on maturation and germination (see root and leaf size in Figure 2) than LS medium without PGRs. It may be suggested that maturation and dormancy exists up to some extent, and exogenous supplementation of GA$_3$ in the regeneration medium had an accelerating effect on the maturation of the bulblets. Rooted bulblets were then acclimatized with a high rate of survival.

Somatic embryogenesis is a process having several advantages over organogenesis, such as the probable single cell origin, which reduces somaclonal variations in several geophytes and is applicable for medium- or long-term storage in sodium alginate beads (Ballester et al., 1997; Patel et al., 2000; Gurel and Gurel, 2014). Heavy collection of bulbs from their natural habitats and their use for breeding propagation purposes could have negative impacts on the plant species. Therefore, for the first time in an ornamental species, sodium alginate encapsulation was achieved using immature bulblets without a loss in vitality at low temperature for 10 weeks. Encapsulation of bulblets with a biodegradable sodium alginate matrix in soil might be a promising approach for large-scale bulb production. It is noteworthy to mention that encapsulated miniature bulblets can provide new insight towards minimizing the storage and transportation cost in the market place for horticultural and ornamental practices. Although Karamian et al. (2011) reported an encapsulation technique using protoplasts of *M. armeniacum* only for somatic embryo induction, we performed encapsulation using immature bulblets, followed by their plantation in soil after a long storage period at 4 °C. However, further studies are necessary for encapsulation methods to develop desiccation treatments, as seen in zygotic embryos under a rough seed coat, to reduce the moisture content and accumulation of storage products in the embryo or in the endosperm (Winkelmann et al., 2004). On the other hand, for the growth and development of the bulblets, soil must be watered frequently and must be under control against pest and fungal diseases until the bulblets adapt to nonaxenic conditions (observed data).

In conclusion, the present study described a simple and effective protocol for the commercially important ornamental species *M. armeniacum* via somatic embryogenesis. Since *M. armeniacum* is one of most commonly cultivated geophytes, a rapid and simple regeneration system would simplify the previous attempts at achieving the formation of somatic embryos at high levels of quality and quantity. Thus, it is plausible to use encapsulation technology for embryos or immature bulblets. Encapsulation of the immature bulblets into a sodium alginate matrix might be a useful technique

for the long-term storage of plants having economic importance. This protocol could not only be used for a large-scale production, but also for ex situ conservation of various heavily collected *Muscari* species. On the other hand, direct planting of the beads in the soil contributes a new understanding towards conservation, storage, and transportation practices underlying the production of artificial seeds in the near future.

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