In vitro micropropagation from immature embryos of the endemic and endangered Muscari muscarimi Medik.

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1. Introduction

Muscari muscarimi Medik., which belongs to the family Liliaceae, is a geophyte endemic to Turkey. Flowers are musk-scented, narrowly oblong-ovoid, initially violaceous, and dirty grayish-white or glaucescent at anthesis. It has 3–6 linear-lanceolate, grayish-green leaves per plant and bulbs of 2–4 cm in diameter. It has a natural distribution in the Antalya and Denizli provinces of Turkey at altitudes of up to 1920 m (Davis, 1984). Extensive habitat destruction in these provinces threatens the existence of the species; thus, it was represented in the endangered categories (Ekim et al., 2000). Collection of M. muscarimi bulbs from their natural habitats is prohibited in Turkey, in accordance with international agreements for the protection of endangered geophytes. Muscari species can be propagated from seeds or bulbs (Nasırcılar et al., 2011; Arslan et al., 2012), but it takes 4–5 years to develop a flowering plant from seed. The propagation ratio of bulbs is also extremely low as bulbs develop only 1–2 bulblets in a 5–6-year period. This low propagation ratio limits the large-scale cultivation of M. muscarimi, a plant that has great potential for the ornamental and perfume industries because of its purple-white flowers with their intense musky fragrance.

Endemic and endangered plants can efficiently be propagated and conserved with in vitro cultural methods (Corral et al., 2011). In vitro bulblet production in many geophytes has been reported previously from different explants (Ziv and Lilien-Kipnis, 2000; Mori et al., 2005; Khosravi et al., 2007; Ascough et al., 2009; Liu et al., 2012) including immature embryos (Mirici et al., 2005; Nasırçılar et al., 2011). Embryos may not be the best explants for propagation due to segregation. However, cross-pollination in Muscari species is known to be low, and propagation in wild flora mostly occurs from seed as bulblet production from the main bulbs is extremely low. Moreover, there is no developed cultivar in M. muscarimi, and bulblet variation is not so important in trade. Therefore, any regenerative...

Abstract: An efficient in vitro bulblet production procedure from immature zygotic embryos of endemic and endangered Muscari muscarimi Medik. was described in the current study. Zygotic embryos were first isolated from immature seeds and cultured on different nutrient media compositions supplemented with various combinations of α-naphthalene acetic acid (NAA), picloram, dicamba, 6-benzylaminopurine (BAP), and thidiazuron (TDZ). The best bulblet regeneration (59 bulblets per explant) was achieved in Murashige and Skoog (MS) medium containing 4 mg/L BAP and 0.5 mg/L NAA after 1 year of culture initiation. Regenerated bulblets were then transferred into MS medium without plant growth regulators for rooting. Bulblets produced well-developed root systems and increased their size on this medium after 2 months. All rooted bulblets were successfully transplanted into a potting mixture and acclimatized to ambient conditions.

Key words: Muscari muscarimi Medik., immature embryo, tissue culture
organs including immature embryos can be an alternative source for the propagation of endemic and endangered species. Here we describe an efficient in vitro bulblet regeneration protocol from immature zygotic embryos of the endemic and endangered *M. muscarini*, which may help field cultivation and germplasm conservation of the species.

2. Materials and methods

2.1. Plant material and sterilization

Bulbs of *M. muscarini* were collected from the natural habitat of Antalya Province, Turkey, in April and were planted in September in the botanical garden of the Field Crops Department of Ankara University. Following the flowering, newly developed fruits containing immature embryos (approximately 1 mm in length) were harvested in May. For surface sterilization, seeds were removed from the fruits and immersed in a 25% commercial bleach solution containing 6% sodium hypochlorite (Axion). The seeds were kept in this solution for 20 min and were then rinsed 3 times with sterile water.

2.2. Immature embryo culture

Immature embryos were easily isolated from the seeds by squeezing with forceps and cultured in vitro using 2 different protocols.

In the first protocol, medium preparation and in vitro culture were carried out as described by Özcans (2002) and Mirici et al. (2005). Isolated, immature embryos were first placed on induction medium containing N6 mineral salts and vitamins (Chu et al., 1975), 200 mg/L casein hydrolysate, 2.3 mg/L L-proline, 2% sucrose, 0.7% agar (Duchefa, P 1001), and 1, 2, 4, and 8 mg/L picloram or 5, 10, and 15 mg/L dicamba and kept at 24 °C in the dark for 3 weeks. After callus development, immature embryos were transferred to a proliferation medium consisting of induction medium with 3% mannitol. The explants were subcultured into newly prepared proliferation medium periodically in 2-week intervals. During subculture, large calli were divided into small pieces. After somatic embryo formation in proliferation media, explants were transferred to maturation medium including Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium and 6% sucrose with no auxins. On maturation media, somatic embryos were gradually exposed to light in 16-h photoperiods. Under these conditions, development of small bulblets took almost 4–5 weeks. The groups of bulblets were then removed from callus tissue and transferred to MS medium supplemented with 2% sucrose in Magenta GA-7 vessels.

In the second protocol, immature embryos were cultured on MS medium containing 0.5–4.0 mg/L 6-benzylaminopurine (BAP) and 0.5–2.0 mg/L a-naphthalene acetic acid (NAA) or 0.25–2.0 mg/L thidiazuron (TDZ), 3% sucrose, and 0.7% agar in petri dishes and were subcultured monthly. After 4 months of culture initiation, regenerated tissues were subdivided and transferred to the fresh medium in Magenta GA-7 culture vessels.

The pH of all media was adjusted to 5.6 and media were autoclaved for 20 min. Unless otherwise stated, all cultures were incubated at 24 °C under cool white fluorescent light (35 µmol photons m⁻² s⁻¹) with 16-h (day)/8-h (night) photoperiods.

2.3. Rooting and ex vitro culture

Regenerated bulblets were finally teased apart from the bearing tissue and were allowed to root in MS medium containing 3% sucrose and 0.7% agar. The rooted bulblets were then transferred to potting mixture containing peat moss, vermiculite, and perlite (1:1:1). Acclimatization to ambient conditions was carried out in pots covered with plastic bags under greenhouse conditions at 18–20 °C.

2.4. Statistical analysis

Each treatment had 3 replicates and all experiments were repeated at least once. Significance was determined by analysis of variance (ANOVA), and the differences between the means were compared by Duncan's multiple range tests using MSTAT-C statistical software (Michigan State University). Data given as percentages were subjected to arcsine (√X) transformation (Snedecor and Cochran, 1967) before statistical analysis.

3. Results

In the first protocol, immature embryos swelled and produced compact embryogenic calli after 3 weeks of culture in induction medium. Transfer of these calli to proliferation medium and subculture every 2 weeks resulted in prolific somatic embryo development (Figure 1a). Bulblet formation from these embryos was achieved in maturation medium after 4–5 weeks of explants being transferred to a 16-h light photoperiod (Figure 1b). After 6 months, these bulblets were finally transferred to MS medium supplemented with 20 g/L sucrose. Serial subcultures of regenerated bulblets in this medium increased the volume of bulblets considerably (to approximately 8–10 mm in diameter) (Figure 1c). The frequency of bulblet regeneration varied in different concentrations of picloram or dicamba in N6 medium (Figure 2). Although bulblet development was not obtained in N6 medium containing 1 mg/L picloram, the highest bulblet regeneration frequency (73% with 15 bulblets per explant) was recorded on N6 medium containing 4 mg/L picloram.

In the second protocol, immature embryos developed large calli on MS medium containing different concentrations of BAP and NAA after 2 months of culture initiation (Figure 3a) with varying amounts of shoots. After 4 months of culture, further development
of shoots led to the formation of bulblets at the base of shoots (Figures 3b and c). The rate of bulblet regeneration ranged between 20% and 93% under a 16-h photoperiod and between 40% and 100% under complete darkness. The maximum number of bulblets per explant was induced on MS medium containing 4 mg/L BAP and 0.5 mg/L NAA in a 16-h day length or in complete darkness (Table). TDZ levels over 0.25 mg/L reduced bulblet regeneration. The highest bulblet production (23% bulblet regeneration frequency with 15 bulblets per explant) was obtained from MS medium supplemented with 0.25 mg/L TDZ (Figure 4).

Figure 1. In vitro bulblet regeneration from immature zygotic embryos of *M. muscarimi* in the first protocol: (a) development of embryogenic callus on proliferation medium containing 4 mg/L picloram after 1 month in culture, (b) prolific shoot regeneration on maturation medium after 6 months in culture, (c) development of shoots into bulblets on MS medium with 20 g/L sucrose after 10 months in culture.

Figure 2. Bulblet regeneration from immature zygotic embryos of *M. muscarimi* on N6 medium supplemented with different concentrations of growth regulators. Values with the same letters are not significantly different at P < 0.05. 1: From immature embryos that produced bulblets.
Table. Bulblet regeneration from immature zygotic embryos of *M. muscarimi* on MS medium supplemented with various concentrations of BAP and NAA in a 16-h photoperiod or complete darkness.

<table>
<thead>
<tr>
<th>Plant growth regulators (mg/L)</th>
<th>Explants producing bulblets (%)</th>
<th>Mean number of bulblets&lt;sup&gt;1&lt;/sup&gt;</th>
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<tr>
<td>BAP NAA</td>
<td>16-h photoperiod</td>
<td>Complete darkness</td>
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<td>1 -</td>
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<sup>1</sup> From immature embryos that produced bulblets.

<sup>2</sup> Values within a column followed by the same letters are not significantly different at P < 0.05.

Figure 3. In vitro bulblet regeneration from immature zygotic embryos of *M. muscarimi* in the second protocol: (a) morphogenic callus formation after 2 months, (b) prolific shoot regeneration after 8 months, (c) development of bulblets after 12 months in culture on MS medium supplemented with 4 mg/L BAP and 0.5 mg/L NAA.

Figure 4. Bulblet regeneration from immature zygotic embryos of *M. muscarimi* on MS medium supplemented with different concentrations of TDZ. Values with the same letters are not significantly different at P < 0.05. <sup>1</sup> From immature embryos that produced bulblets.
Rooting of bulblets was achieved in MS medium without growth regulators. Almost all bulblets developed good root systems on this medium after 2 months (Figure 5a). Rooted bulblets were then transplanted into pots containing a peat moss, vermiculite, and perlite mixture (1:1:1) and acclimatized to ambient conditions in greenhouse at 18–20 °C. All transferred plantlets survived in the pots (Figure 5b).

4. Discussion
Bulb scales are the most common explants used for in vitro propagation of geophytes (Mirici et al., 2005; Mori et al., 2005). However, the use of bulbs as starting material is often associated with heavy bacterial or fungal contamination (Langens-Gerrits et al., 1998; Ziv and Liliens-Kipnis, 2000; Karagüzel et al., 2012; Liu et al., 2012). Moreover, collection of bulbs from wild flora and their use for propagation purposes could have negative impacts on endangered plant species. Apart from bulb scales, immature zygotic embryos have also been used successfully as an alternative explant source for the micropropagation of endangered Sternbergia fischeriana (Mirici et al., 2005), Muscari azureum (Uranbey, 2010b), and M. mirum (Nasırcılar et al., 2011) species, and contamination was not reported, as in the present study. Genetic variation may be the only weakness of bulblet production from immature zygotic embryos. However, cross-pollination in M. muscarimi is known to be low, and propagation in wild flora mostly occurs from seed without any visible phenotypic variations (personal comm., Prof. Neşet Arslan). Therefore, use of immature zygotic embryos for the micropropagation of M. muscarimi may have great value.

In the first protocol, immature zygotic embryos of M. muscarimi were cultured on N6 medium containing dicamba and picloram for bulblet regeneration. Induction of bulblets was not recorded on N6 medium without growth regulators. The addition of plant growth regulators into the culture media was important for in vitro regeneration of M. muscarimi. The concentration of dicamba or picloram in the culture medium was found to have a critical role for bulblet regeneration, such that picloram was more potent than dicamba. The highest bulblet regeneration was obtained on N6 medium containing 4 mg/L picloram. The promotory effect of picloram and dicamba on callus formation and bulblet regeneration has also been reported previously for many bulbous plant species (Xu et al., 2000; Mori et al., 2005, Khosravi et al., 2007; Ault and Siqueira, 2008). Khosravi et al. (2007) investigated the effect of various concentrations of picloram on direct somatic embryogenesis in Lilium and indicated that MS medium supplemented with 2 mg/L picloram was the most effective treatment for induction of embryogenesis. Ault and Siqueira (2008) also observed that shoot formation varied significantly in response to dicamba, picloram, or 2,4-D concentrations in Lilium michiganense. Nasırcılar et al. (2011) could not obtain any callus or regeneration on MS medium supplemented with picloram in Muscari mirum. Such differences may result from the genotype, explants, or concentrations of growth regulators used in different studies.

The results of the second protocol suggested that the development of bulblets was also promoted by BAP and NAA in MS medium. Such findings correspond well with the results of Nakano et al. (2005), Özel et al. (2007), and Nasırcılar et al. (2011) reporting stimulated bulblet regeneration in Muscari armeniacum, M. macrocarpum, and M. mirum with BAP and NAA supplementation. In the current study, the maximum number of bulblets per explant was induced on MS medium containing 4 mg/L BAP and 0.5 mg/L NAA (59 bulblets per explant). Mirici et al. (2005) reported that MS medium supplemented

Figure 5. In vitro rooting and acclimatization of M. muscarimi: (a) root formation on regenerated bulblets, (b) development of M. muscarimi plants from in vitro regenerated bulblets after 2 months in greenhouse.
with 4 mg/L BAP and 0.25 mg/L NAA induced 81.2 bulblets per immature embryo explant in *Sternbergia fischeriana*, and Nasrçilar et al. (2011) reported that immature embryos of *M. mirum* produced 9.23 bulblets on MS medium containing 2 mg/L BAP and 0.25 mg/L NAA. Utilization of TDZ in MS medium reduced bulblet regeneration compared to BAP and NAA. Increasing TDZ concentration decreased bulblet production. Similar to these findings, TDZ did not effectively promote bulblet regeneration in *M. acheri* and *M. azureum* (Uranbey, 2010a, 2010b; Uranbey et al., 2010).

In conclusion, culturing immature embryos on MS medium containing BAP and NAA is an effective method for in vitro propagation of *M. muscarimi*. In the present culture system it took 12–14 months from initial culture to bulblet regeneration and transfer of bulblets to fields successfully. This method can be used for propagation and conservation of endemic and endangered *M. muscarimi*.

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**References**


