***IN VITRO* MICROPROPAGATION OF THE ENDEMIC AND ENDANGERED *MUSCARI MUSCARIMI* MEDIK.**

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**Abstract**

A procedure is described for the efficient *in vitro* bulblet production from immature zygotic embriyos of endangered and endemic *Muscari muscarimi* Medik. Immature zygotic embryos were isolated from immature seeds and cultured on MS or N6 medium containing various combinations of auxins (α-naphthaleneacetic acid, Picloram, Dicamba) and cytokinins (6-benzylaminopurine, thidiazuron). The highest bulblet regeneration was achieved (59 bulblets/explants) from immature embryos on Murashige and Skoog (MS) medium containing 4 mg l-1 6-benzylaminopurine (BA) and 0.5 mg l-1 α-naphthaleneacetic acid (NAA) after one year of culture. Regenerated bulblets were transferred to MS medium without plant growth regulators for rooting. All bulblets produced well-developed root system and increased in size on this medium after 2 months. Rooted bulblets were transplanted to a potting mixture and acclimatized to ambient conditions with a 100% success.

**Key words:** *Muscari* *muscarimi*, in vitro micropropagation, immature embryo

**Running title**: Micropropagation of *Muscari* *muscarimi*

**INTRODUCTION**

Habitat destruction has resulted in disappearance of many plant species from the earth forever and many more face extinction. Likely, endemic and endangered M*uscari muscarimi* Medik. (musky grape hyacinth) belonging to *Liliaceae* family and growing in natural habitats of Antalya and Denizli provinces of Turkey at altitudes up to 2000 m (Ekim 2000, Tubives 2007) is also threatened by complete extinction. Therefore, collection of *M. muscarimi* bulbs from the natural habitats is forbidden in Turkey, in accordance with international agreements for the protection of endangered geophytes. Moreover, the natural propagation rate of *M. muscarimi,* like most geophytes is relatively low (Kim and De Hetrogh 1997, Arslan et al. 2002, Mirici et al. 2005) and it takes 4 or 5 years to develop a flowering plant from seed. Propagation ratio of bulbs are also low as some bulbs do not develop new bulblets and some produces only 1-3 bulblets in a 3 years period. This low propagation ratio limits the large-scale cultivation of *M. muscarimi* which has great potential in ornamental and perfume industry because of its attractive purple-white flowers with an intense musky fragrance.

Besides conventional methods of propagation, endemic and threatened plants could efficiently be propagated and conserved with *in vitro* cultural methods, which have low impact on wild populations with a minimum of plant material. *In vitro* bulblet production in many geophytes has been reported previously from different explants. Recently, in our laboratories we first time used the immature zygotic embryos for *in vitro* micropropagation of endangered geophyte *Sternbergia fischeriana* and produced 85 bulblets from single immature embryo explant (Mirici et al. 2005). However, we could not transfer the bulblets to soil successfully. The present study aimed to establish a strategy for efficient *in vitro* bulblet regeneration in *M. muscarimi* using immature zygotic embryos and successful transplantation of bulblets to soil. *In vitro* bulblet production in *M. muscarimi* has not been reported previously.

**MATERIALS AND METHODS**

**Plant material and disinfection**

Bulbs of *M. muscarimi* collected from the natural flora of Antalya were planted in the botanical garden of the Field Crops Department of University of Ankara for immature embryo supply. Immature fruits containing immature embryos at appropriate physiological age of development were harvested from these plants in early spring. For surface sterilization, seeds were removed from these fruits and immersed in 25% commercial bleach (Axion) for 20 minutes, then rinsed three times with sterile water.

**Immature embryo culture**

Immature embryos (approximately 1mm in length) were isolated by squeezing the seed with forceps and cultured *in vitro* using two different protocols as described earlier (Mirici et al. 2005). In the first protocol, medium preparation and *in vitro* culture work were carried out as described earlier (Bronsema et al. 1997, Özcan 2002, Mirici et al. 2005) with some modifications. Isolated immature embryos were first placed on ‘induction medium’ containing N6 minerals and vitamins (Chu et al. 1975), 200 mg l-1 casein hydrolysate, 2.3 mg l-1 L-Proline, 2% sucrose, 0.7% agar and 1-8 mg l-1 Picloram or 5-15 mg l-1 Dicamba and kept at 24oC in the dark for 2 weeks. Immature embryo explants developing callus were then transferred to ‘proliferation medium’ (induction medium with 3% mannitol) and subcultured onto fresh medium at 2 week intervals. During subculture large callus explants were subdivided into small sizes. After prolific somatic embryo formation on proliferation media, explants were transferred to ‘maturation medium’ consisted of MS medium, 6% sucrose and without auxins. On maturation media, somatic embryos were gradually exposed to light with 16-h photoperiod and developed into small bulblets after 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 MSO medium containing 0.5-4 mg l-1 6-benzylaminopurine (BA) and 0.5-2 mg l-1 α-naphthaleneacetic acid (NAA) or 0.25-2.0 mg l-1 thidiazuron (TDZ) in Petri dishes and subcultured regularly every month. After 4-5 months of culture initiation, callus tissue regenerating buds were subdivided and transferred to the same medium in Magenta GA-7 culture vessels. MSO medium consisted of Murashige and Skoog’s (MS) mineral salts and vitamins (Murashige and Skoog 1962), 3% sucrose and 0.7% agar. 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 photoperiod.

**Rooting and ex vitro culture**

Regenerated bulblets were finaly teased apart from the bearing tissue and rooted MSO medium. The rooted bulblets were then transferred to potting mixture containing peat moss, vermiculite and perlite (1:1:1) and acclimatized to ambient conditions in pots covered with a plastic bag and later established in greenhouse at 18-20oC.

**Statistical analysis**

Each treatment had three replicates containing 5-10 explants in culture vessels 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 an MSTAT-C computer program (Michigan State University). Data given as percentages were subjected to arcsine (√X) transformation (Snedecor and Cochran 1967) before statistical analysis.

**RESULTS**

In the first protocol, immature embryos swelled and produced compact embryogenic callus (Figure 1a) after one month of culture on induction medium. When the explants were transferred to proliferation medium and subcultured onto fresh medium every two weeks, prolific shoot formation occurred on this callus tissue. These shoots developed into bulblets after 4-5 weeks of explant transfer to maturation medium in 16 h light photoperiod (Figure 1b). These bulblets were finally transferred to MS medium supplemented with 20 g l-1 sucrose and serial subcultures of regenerated bulblets until 10 months increased the volume of bulblets considerably (Figure 1c). The frequency of bulblet regeneration on different concentrations of picloram or dicamba in N6 medium varied (Table 1). Higher bulblet regeneration was obtained on N6 medium containing picloram compared to dicamba. Maximum bulblet regeneration frequency of 73% with 15 bulblets per explant was recorded on N6 medium containing 4 mg l-1 picloram.

In the second protocol, immature embryos developed into large calli on MS medium containing different concentrations of BAP and NAA after two months (Figure 1d), with varying amount of shoots after 6 months of culture (Figure 1e). Further development of shoots led to the formation of bulblets at the base of shoots after 12 months of culture (Figure 1f). Rate of bulblet regeneration frequency ranged 20-93% under 16 h day length and 40-100 % under darkness respectively. The maximum number of bulblets per explant was induced on MS medium containing 4 mg l-1 BAP and 0.5 mg l-1 NAA in either 16 h day length or complete darkness. Photoperiod conditions did have a considerable effect on bulblet regeneration (Table 2). Utilization of TDZ in MS medium reduced the bulblet regeneration compare to BAP and NAA and bulblet regeneration was inhibited in MS medium containing 0.5 mg l-1 TDZ (Table 3).

**Rooting and Ex vitro culture**

Regenerated bulblets were separated from each other and transferred to MSO medium for rooting and further development. Nearly all bulblets rooted and produced a very good root system on this medium after 2 months (Figure 1g). The rooted bulblets were then transplanted to potting mixture and acclimatized to ambient conditions with a 100% success. *In vitro* regenerated bulblets were finally established well in greenhouse at 18-20 oC (Figure 1h).

**DISCUSSION**

Usually, *in vitro* methods of multiplication of ornamental bulbous plants make use of clonal propagation of plants with superior characteristics; as cloning is essential for establishing a cultivar in allogamous plants. On the other hand, only certain genotypes are selected by clonal propagation. Use of immature zygotic embryos in tissue culture of bulbous ornamental plants is not desirable due to expected genetic variation; which may end up with inferior genotypes. However, For conservation of endemic and endangered plants like *M. muscarimi*, it is desirable to conserve them through any of the available technology with a little damage to their natural habitats. As such, propagation through zygotic embryos or seeds could be used as an important source to maintain the genetic diversity in *M. muscarimi*.

Use of immature seeds or zygotic embryos, which are obtained from encapsulated fruit covers as in this study, is not associated with contamination problems. They are easy to sterilize and provide an excellent source of contamination-free explants. The use of bulbs as starting material is often associated with heavy bacterial or fungal contaminations (Langens-Gerrits et al. 1998, Ziv and Liliens-Kipnis 2000, Parmaksiz and Khawar 2006), which have also a negative impact on the population of already endangered species. Therefore, it was considered vital to use immature zygotic embryos in this study. Previously immature zygotic embryos have been used in *Stenbergia fischeriana* (Mirici et al. 2005), lesser burnet (Çöçü et al. 2003), Hungarian vetch (Sancak et al. 2000), corn (Özcan 2002), and *Narcissus confusus* (Selles et al. 1999), sainfoin (Özcan et al. 1996), pea (Özcan et al. 1993), The present work demonstrated high frequency of adventitious bulblet regeneration from immature zygotic embryos of *M. muscarimi* on N6 and MS media containing dicamba, picloram, BAP and NAA. No induction of bulblets was recorded on MS medium in contradiction to Karamian et al. (2011); who showed that *M. neglectum* was easily propagated using bulb explants in MS medium without addition of growth regulators.

Cytokinins have been reported to induce bulblet regeneration in *Lilium candidum* solely, or in combination with an auxin (Khawar et al. 2005, Sevimay et al. 2005). The observations in this study would suggest that development of adventitious bulblet is promoted more by BAP and NAA in MS medium; a high nitrogen medium, than on picloram or dicamba in N6 medium; a low nitrogen medium. This is in aggrement with Nasırcılar et al. (2011) who showed that addition of BAP-NAA stimulated plantlets formation in *Muscari* *mirum*. The concentration of dicamba or picloram in the culture medium of first protocol played critical role for adventitious bulblet regeneration, such that picloram was more potent compared to dicamba (Table 1). Superiority of picloram for adventitious bulblet regeneration may be attributed to the ability of plant tissues to metabolize picloram more readily than dicamba. The promotory effect of dicamba and picloram in inducing callus and adventitious bulblet regeneration has been previously reported in many bulbous plant species. Mori et al. (2005) found that seed, bulb scale, leaf, or filament explants cultured on 4.1 *μ*M picloram and cultured in the dark regenerated variable amount of callus. Callus lines showed sustained growth 1 year after the initiation of subculture and produced shoots on a medium without plant growth regulators (PGRs) and a medium containing 22 *μ*M 6-benzyladenine (BA). Shoot regeneration was observed in all genotypes; however only 20 genotypes showed regeneration frequency of over 80%. Most of the regenerated shoots developed into complete plantlets following their transfer to a PGR-free medium. Similarly Pinsan et al. (2000) induced callus from bulb scales of *Lilium longiflorum* cv. Georgia in MS medium supplemented with 5.0 µM picloram. Callus growth was more vigorous in liquid than on a solid medium. When the calluses were transplanted to MS medium with half strength inorganic elements and full strength organic components, the frequency of callus regenerating shoots and number of shoots per callus was higher on the solid than in the liquid medium. However, the shoot forming capacity decreased as the number of subcultures increased. Bulblets regenerated from subcultured calluses were stored at 4 degrees oC for 8 weeks and then transplanted to soil. Ault and Siqueira (2008) observed that shoot formation varied significantly in response to individual dicamba, picloram and 2.4 D concentration in *Lilium* *michiganense*. A maximum of 7.9 shoots per explant was promoted by 4.0 µM K-NAA and 1 µM dicamba respectively. At no stage of bulb development addition of activated charcoal was considered necessary to promote bulblet development as immature embryos showed proliferated development of adventitious bulblets on all media irrespective of the type of combination of growth regulators. The results are in contradiction with Peck and Cuming (1986), who successfully tissue cultured bulbs of *M. armeniacum* by using 1 g l-1 activated charcoal in MS medium.

Culture medium was found to significantly influence callus induction and adventitious bulblet regeneration. Modified many stepped protocol by Bronsema et al. (1997), Özcan (2002) and Mirici et al. (2005) was found inferior to one step protocol using 16 h light with more bulblets regeneration per explant. A range of explants including immature zygotic embryos, immature seeds, stem nodes, bulb scales, leaves, mature seeds, filaments with anther and thin cell layers have been used for *in vitro* bulblet production from bulbous geophytes (Arzate-Fernandez et al. 1997, Nayak et al. 1997, Nhut 1998, Selles et al. 1999, Sage et al. 2000; Wawrosch et al. 2001, Nhut et al. 2002, Paek and Murthy 2002, Lian et al. 2003, Kim et al. 2003, Sevimay et al. 2005, Khawar et al. 2005, Mirici et al. 2005, Parmaksiz and Khawar 2006). This study clearly establish that like other explants, immature zygotic embryos or immature seeds of endangered *M. muscarimi* could also be successfully cultured on suitable combination of auxin and cytokinin to regenearate new bulblets and have great potential for *in vitro* morphogenesis.

It was not necessary to root the bulblets on separate media. Developing bulblets showed variable number of roots on maturation medium. Regenerated bulblets were separated from each other and transferred to MSO medium for rooting and further development. The results are in agreement with Özel et al. (2007), who found that bulblets of *Muscari macrocarpum* could be rooted on MS medium. Nayak and Sen (1995), found that shoots of *Ornithogalum umbellatum* could be rooted in 1/2-strength MS medium. Similarly, Tang et al. (2007), regenerated shoots of *Chirita heterotricha* on half-strength MS medium with 5 g l-1 activated charcoal, 30 g l-1 sucrose. *In vitro* produced secondary bulblets could be transplanted directly to potted soil mixture and were not difficult to acclimatize, in agreement with Naik and Nayak (2005).

Availability of *in vitro* propagation techniques are of particular importance for *M. muscarimi*, since multiplication of the plant by seeds is very difficult. Under natural condition, *M. muscarimi* takes two years from seeds to develop leaves, and two or three years more for the bulbs to mature to flower. If the period from seeding to bulb formation is reduced, it will reduce time to flowering and seed production and accelerate the rate of plant multiplication. This is desired in the conservation and breeding of endangered plants. We found that the culture of zygotic embryos on MS medium containing BAP-NAA is an effective method to induce rapid germination and growth of *M. muscarimi*. In our culture system, it took 8 months from initial culture to bulblet regeneration and transfer of bulblets to fields successfully. This method is useful to propagate *M. muscarim*i and conserve this endangered plant. This *in vitro* regeneration strategy should have a role in long-term conservation and management of this critically rare and endangered species.

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**Tables**

Table1. Bulblet regeneration from immature zygotic embriyos of *M. muscarimi* on N6 medium supplemented with different concentrations of picloram and dicamba after 12 months in culture.

|  |  |  |  |
| --- | --- | --- | --- |
| Growt regulators (mg l-1) | | Explants producing bulblets (%) | Mean number of bulblets per explant**2** |
| Picloram | Dicamba |
| 1 | - | 0 d**1** | 0 b |
| 2 | - | 27 bc | 6 b |
| 4 | - | 73 a | 15 a |
| 8 | - | 53 ab | 4 b |
| - | 5 | 23 bc | 2 b |
| - | 10 | 17 cd | 2 b |
| - | 15 | 30 bc | 4 b |

**1**Values within a column followed by different letters are significantly different at the 0.05 level.

**2** From immature embryos which produced bulblets.

Table 2. Bulblet regeneration from immature zygotic embryos of *M. muscarimi* on MS medium supplemented with various concentrations of BAP and NAA in 16-h photoperiod or complete darkness after 12 months in culture.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Growt regulators  (mg l-1) | | Explants producing bulblets (%) | | Mean number of bulblets per explant**2** | |
| BAP | NAA | 16-h photoperiod | Complete darkness | 16-h photoperiod | Complete darkness |
| 1 | - | 93a**1** | 80ab | 27bc | 13d |
| 2 | - | 20 c | 100 a | 42 b | 43 b |
| 4 | - | 53 abc | 67 abc | 16 c | 17 d |
| 0.5 | 1 | 43 bc | 40 c | 12 c | 20 d |
| 0.5 | 2 | 80 ab | 93 ab | 43 b | 25 d |
| 1 | 1 | 53 abc | 67 bc | 17 c | 22 d |
| 2 | 0.5 | 87 a | 90 ab | 15 c | 34 c |
| 2 | 1 | 88 ab | 40 c | 7 c | 12 d |
| 4 | 0.5 | 80 ab | 63 bc | 59 a | 55 a |
| 4 | 1 | 85 ab | 100 a | 31 bc | 48 ab |

**1**Values within a column followed by different letters are significantly different at the 0.05 level.

**2** From immature embryos which produced bulblets.

Table 3. Bulblet regeneration from immature zygotic embriyos of *M. muscarimi* on MS medium supplemented with different concentrations of TDZ after 12 months in culture.

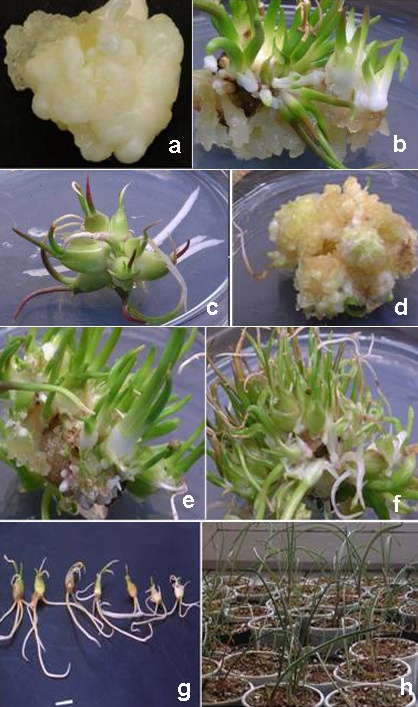
|  |  |  |
| --- | --- | --- |
| TDZ (mg l-1) | Explants producing bulblets (%) | Mean number of bulblets per explant**2** |
| 0.25 | 23 a**1** | 15 a |
| 0.5 | 0 c | 0 b |
| 1 | 17 b | 7 ab |
| 2 | 7 c | 1 b |

**1**Values within a column followed by different letters are significantly different at the 0.05 level.

**2** From immature embryos which produced bulblets.

**Figure Legend**

**Fig. 1.** *In vitro* bulblet regeneration from immature zygotic embryos of *Muscari muscarimi*. (a) Development of embryogenic callus on induction medium containing 4 mg/l picloram after one month in culture. (b) Prolific shoot regeneration on proliferation medium containing 4 mg/l picloram after 6 month in culture. (c) Development of shoots into bulblets on maturation medium after 10months in culture. (d) Morphogenic callus formation after 2 months, (e) prolific shoot regeneration after 8 months and (f) developments of bulblets after 12 months in culture on MS medium supplemented with 4 mg/l BAP nad 0.5 mg/l NAA. (g) Root formation on regenerated bulblets and (h) developments of *M. muscarimi* plants from *in vitro* regenerated bulblets in green house.

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