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Full Length Research Paper

In vitro bulblet regeneration from immature embryos of *Muscari azureum*

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A high frequency bulblet regeneration was achieved for endemic and endangered ornamental plant *Muscari azureum* using immature embryos. Immature embryos of *M. azureum* were cultured on callus induction medium consisting of N_6 mineral salts and vitamins, 400 mg/L casein + 40 g/L sucrose + 2 g/l L-proline, 2 mg/L 2,4-D and 2 g/L gelrite. Then, embryogenic callus clusters were transferred to *bulblet induction* medium consisting of MS mineral salts and vitamins containing different concentrations and combinations of N^6-benzylamino-purine (BAP), kinetin (KIN), thidiazuron (TDZ), zeatin, indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), 30 g/L sucrose and 7 g/L agar. Prolific bulblets multiplication (over 13 bulblets/embryo) was achieved from immature embryos after 5 - 6 months of culture initiation. Well-developed bulblets were excised and individually rooted on ½ strength MS medium supplemented with 1 mg/l IBA, 0.5 g/l activated charcoal, 20 g/l sucrose and 6 g/l agar and acclimatized.

Key words: *Muscari azureum*, bulblet, micropropagation, immature embryo.

INTRODUCTION

The genus *Muscari* Mill., which comprises about 50 species distributed from the temperate European and the Mediterranean to Central Asia, belongs to the subfamily *Hyacinthoideae* Link. of the family *Hyacinthaceae* Batsch (Speta, 1998a, b). It is an endemic and endangered species of Turkey as it is being threatened by complete extinction in the future. Low propagation rate in nature and irregular collection of bulbs of *M. azureum* from their habitat also hampers the cultivation of the species. Plant tissue culture techniques is an influential tool, which can be engaged as an alternative to the conventional method of vegetative propagation with the objective of enhancing the rate of multiplication of desired genotypes (Hussey, 1986; Murashige, 1990; Naik and Nayak, 2005). Different explants types can been used for *in vitro* micropropagation of geophytes from a range of explants including bulb scales, shoot tips, perianth, stem nodes, root, mature leaves and mature seeds to thin cell layers. However, there are limited protocols about micro-propagation of geophytes using immature embryo as starting material. Immature embryos can be exquisite source of explant to overcome explant contamination originating from underground storage organs. Also, shoot regeneration for *M. azureum* via either organogenesis or somatic embryogenesis has not yet been reported. The aim of the study is the development of a high frequency *in vitro* bulblet regeneration protocol for ornamental plant *M. azureum* using immature embryos.

Abbreviations: BAP, N^6-Benzylamino-purine; **MS,** Murashige and Skoog medium; **NAA,** α-naphthaleneacetic acid; **KIN,** kinetin; **TDZ,** thidiazuron; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid.

MATERIALS AND METHODS

Plant material and surface sterilization

Bulbs of *M. azureum* were collected from wild flora of Amasya, Kayseri and Ankara provinces of Turkey in April (Figure 1a) and planted in a Greenhouse. Immature fruits of *M. azureum* were harvested in May - June. Seeds were kept out of fruits and surface-sterilized by treatment for 2 min in 95% ethanol then in 40% commercial bleach (Axion) for 20 min and then rinsed three times

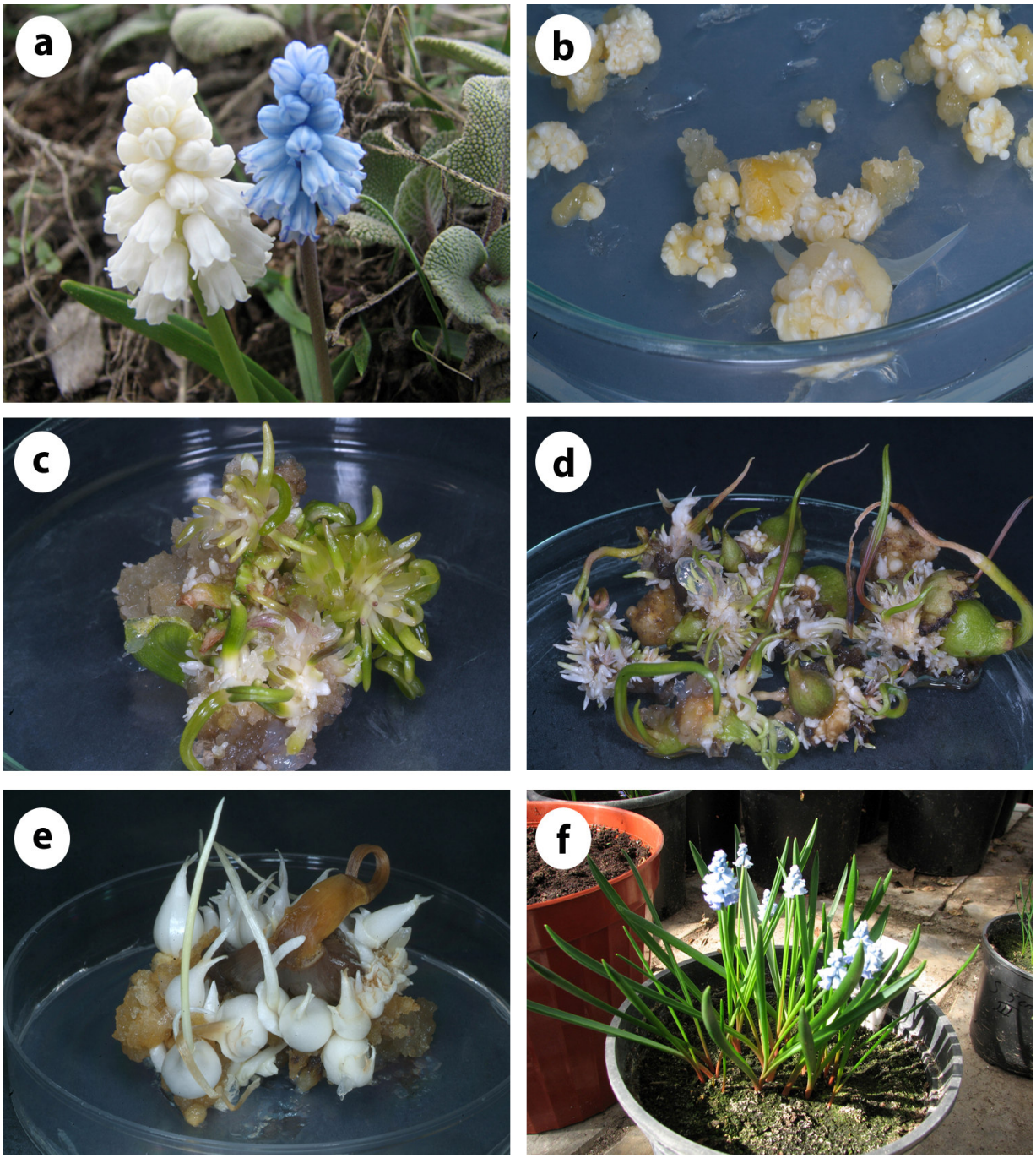

Figure 1. Bulblet production from immature embryos of *M. Azureum*. (a) *M. azureum* growing in its natural habitat (Kayseri province); (b) embryogenic callus formation on callus induction medium after 8 - 10 weeks in culture; (c) shoot and bulblet regeneration on bulblet induction medium; (d) prolific bulblet induction on MS medium supplemented with 1.0 mg/L BAP and 0.25 mg/L IAA; (e) well developed bulblet maturation medium after 9 months in culture and (f) acclimatization of rooted bulblets in pots containing compost and peat mixture.

with sterile water.

Isolation of immature embryos

Following the removal of immature seeds from fruits and surface sterilization, the seed coat encasing the immature embryo was peeled away and the seed was squeezed hard using forceps until the immature zygotic embryo (approximately 0.5 - 1.0 mm in length) was released as described by Mirici et al. (2005). Immature embryos cultured on callus induction medium consisted of N_6 mineral salts and vitamins (Chu, 1978), 400 mg/L casein + 40 g/L sucrose + 2 g/l L-proline, 2 mg/L 2,4-D and 2 g/L gelrite for 42 - 70 days. Then, they were transferred to bulblet induction medium which consisted

of MS (Murashige and Skoog, 1962) mineral salts and vitamins containing different concentrations and combinations of N^6-benzylamino-purine (BAP), kinetin (KIN), thidiazuron (TDZ), zeatin, indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA) + 30 g/L sucrose and 7 g/L agar.

Culture conditions and statistical analysis

Basal media salts, vitamins, sucrose, agar and growth regulators were obtained from Duchefa Biochem B.V. Netherlands. The pH of the medium was adjusted to 5.7 with 1 N NaOH or 1 N HCl before autoclaving at 121°C, 117.679 kPa for 20 min. All cultures were kept at 24 ± 1°C under cool white fluorescent light (35 µmol m^{-2} s^{-1}) with 16-h photoperiod. All growth regulators were filter-sterilized using a Milipore filter (0.22 µm pore size) and added to hot autoclaved medium before dispensed into culture tubes. Immature embryos were cultured on 35 ml of bulblet induction media in glass Petri dishes (10 x 100 mm) and subcultured several times on the same media in Steril Vent Container at 2 or 3 weeks interval until prolific formation of bulblets. All bulblets were subcultured and cultivated singly, double or triple according to the size of bulblet cluster on the same media.

Rooted bulblets (5-10 mm) were removed from their culture vessels and transferred to pots containing compost grown in a cultivation cabinet at 20 - 22°C. 10 - 20 embryos per petri dishes per sterile Vent Container were used in the study for each replication. Each treatment had four replicates and all experiments were repeated twice. 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 computer programme (Michigan State University). Data given in percentages were subjected to arcsine (√X) transformation (Snedecor and Cochran 1967) before statistical analysis.

RESULTS AND DISCUSSION

Bulblet regeneration from immature zygotic embryos

Immature embryos were firstly cultured on callus induction medium for 42 - 70 days at 24 ± 1°C and dark conditions. No contamination was observed on immature embryo explants. All of the immature embryo explants were completely covered with morphogenetic callus. Embryogenic callus clusters were induced and was firstly visible on calli within 8-10 weeks in culture (Figure 1b). Embryogenic calli were transferred to bulblet induction medium at the same culture conditions, the petri dishes were covered by soft translucent paper. When embryos were transferred to bulblet induction medium and exposed to low light intensity, somatic embryos formed hard structures. Bulblets and prolific shoot regeneration was seen on these calli within 5-7 weeks. These shoots developed into small bulblets 4 months after culture initiation (Figure 1c). The percentage of explants producing shoots or bulblet and the number of bulblets per embryo were scored after 2 - 3 months, whereas the number of bulblets per explant was recorded after 5 - 6 months of culture initiation. The percentage of explants producing shoots and the number of bulblets per embryo showed that the frequency of bulblet production from different explants was influenced by the levels of BAP x IAA and KIN x IAA concentrations ($p < 0.01$).

The highest percentage of regenerated shoots (75.0%) and the highest number of bulblets per embryo (10.0) occurred with 1.0 mg/L BAP and 0.25 mg/L IAA on tested media containing BAP x IAA combinations. Considering both percentage of explants producing shoots and the number of shoots per embryo, the best shoot multiplication was achieved on a bulblet induction medium supplemented with 1.0 mg/L BAP and 0.25 mg/L IAA for BAP x IAA combinations (Table 1; Figure 1d). Whereas the highest percentage of regenerated shoots (52.5%) was achieved on a range of media supplemented with 2.0 mg/L KIN and 0.50 mg/L IAA on tested media containing KIN x IAA combinations. Also, the highest number of bulblets per embryo (13.25) was obtained from a medium containing 1.0 mg/L KIN and 0.25 mg/L IAA on tested media containing KIN x IAA combinations (Table 2).

Similarly, when different bulblet induction medium containing different concentrations and combinations of TDZ x NAA and Zeatin x NAA were compared, the medium supplemented with 0.05 m/L TDZ and 0.1 mg/L NAA produced the highest number of bulblets per embryo (Table 3.). It was seen that medium containing TDZ and NAA produced bulblets, however, formed bulblets were more vitrified and concave when compared to other media containing the combinations of BAP x IAA and KIN x IAA.

All shoots and bulblets forming on the different media were transferred to bulblet development medium containing MS basal medium supplemented with 20 mg/L mannitol, 500 mg/L casein, 60 g/L sucrose and 2 g/L gelrite. They were subcultured every two weeks onto fresh medium.

After 5-6 weeks, they were transferred to bulblet maturation medium containing MS mineral and vitamins, 20 g/L sucrose, 7 g/L agar. Well-developed bulblets were separated and distributed to new culture vessels for further development. It was seen that some of the bulblets formed onion skin on this medium (Figure 1e).

Well-developed bulblets were excised again and individually rooted on ½ strength MS medium supplemented with 1 mg/L IBA, 0.5 g/L activated charcoal, 20 g/L sucrose and 6 g/L agar. Formation of well-developed and rooted bulblets (approximately 5-10 mm in diameter) was achieved after 9-12 months in culture. Rooted bulblets were pre-treated at + 4°C for 4 - 6 weeks in dark before transferring to compost and peat mixture. Regenerated plants from immature embryos were acclimatized with a 3% survival rate after 2 weeks (Figure 1f).

The type of explant is extremely important in establishing an efficient micropropagation and regeneration system (Koroch et al., 2002; Uranbey et al., 2003; Başalma et al., 2008). High frequency of shoot multiplication and somatic embryogenesis has been obtained previously from immature embryo explants of many other herbaceous plant species such as soybean (Hartweck et al., 1988) pea (Özcan et al., 1993), sainfoin (Özcan et al., 1996), wheat (Özgen et al., 1996) and maize (Bronsema et al., 1997).

Table 1. Influence of BAP and IAA combinatons on explants producing shoots or bulblets and number of shoots per embryo from immature embryos.

Growth regulators (mg/L)		Explants producing shoots or bulblets (%)	Number of bulblets per embryo
BAP	IAA		
0.5	0.25	40.0 b	0.0 c
1.0	0.25	75.0 a	10.0 a
2.0	0.25	45.0 b	4.50 b
0.5	0.50	2.5 c	0.25 c
1.0	0.50	65.0 a	5.75 b
2.0	0.50	37.5 b	5.50 b

*Values within a column followed by different letters are significantly different at the 0.01 probability level using Duncan's multiple range test.

Table 2. Influence of KIN and IAA combinations on explants producing shoots or bulblets and number of shoots per embryo from immature embryos.

Growth regulators (mg/L)		Explants producing shoots or bulblets (%)	Number of bulblets per embryo
KIN	IAA		
0.5	0.25	12.50 c	1.75 cd
1.0	0.25	27.50 b	13.25 a
2.0	0.25	50.0 a	12.0 a
0.5	0.50	0.0 d	0.0 d
1.0	0.50	22.5 bc	3.75 bc
2.0	0.50	52.5 a	4.50 b

*Values within a column followed by different letters are significantly different at the 0.01 probability level using Duncan's multiple range test.

Table 3. Influence of Zeatin x NAA and TDZ x NAA combinations on explants producing shoots or bulblets and number of shoots per embryo from immature embryos.

Growth regulators (mg/L)			Explants producing shoots or bulblets (%)	Number of bulblets per embryo
Zeatin	TDZ	NAA		
0.25		0.1	20.0 bc*	3.50** c
0.50		0.2	52.5 ab	3.53 c
1.0		0.4	0.0 c	0.0 d
2.0		0.8	7.5 b	2.80 cd
	0.05	0.1	70.0 a	10.0 a
	0.10	0.2	67.5 a	9.0 ab
	0.25	0.4	57.5 ab	8.2 b
	0.50	0.8	75.0 a	5.5 c

*,**Values within a column followed by different letters are significantly different at the 0.01 and 0.05 probability level using Duncan's multiple range test, respectively.

There are scarce protocols about micro-propagation of geophytes using immature embryo as initial material. A prolific *in vitro* bulblet production from immature embryos of *Sternbergia fisheriana* and *Ornithogalum platyphyllum* were reported (Mirici et al., 2005; İpek et al., 2009). Immature embryos which have high regeneration capacity may be an alternative explant source for the micro-propagation of geophytes as emphasized by Mirici et al. (2005). Our study showed that the use of immature embryos on *in vitr*o multiplication studies of *M. azureum*

resulted in high frequency of bulblets production. Furthermore, the use of underground storage organs as source causes heavy and fungal contamination of the *in vitro* culture (Langens-Gerrits et al., 1998; Ziv and Lilien-Kipnis, 2000; Mirici et al., 2005). No contamination was observed on immature embryo explants of *M. azureum* in our study. In this context, the present study also indicated that immature embryo explants of *M. azureum* may have an important non-contaminated material for *in vitro* morphogenesis. In earlier studies regarding shoot and bulblet multiplication in *Muscari* species, various auxin and cytokinin combinations were used for shoot and bulblet induction (Deborah, 1985; Peck and Cuming, 1986; Kromer, 1989; Kromer and Kukulczanka, 1992). Superiority of combinations of TDZ and NAA was generally seen on multiplication of *M. azureum* using immature embryo explants; however, the media containing TDZ and NAA produced more vitrified and hollow bulblets.

In conclusion, *M. azureum* have low proliferation ratio in the wild flora and because of low propagation ratio, the commercial propagation of this species is limited. Therefore, development of *in vitro* multiplication of this important endangered and endemic species can be helpful for commercial production and germplasm conservation. Procedures for the production of a new and highly prolific embryogenic culture and bulblet induction system have been developed in *M. azureum*. We also revealed the first report of *in vitro* shoot regeneration and bulblet production from immature embryos of *M. azureum.*

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