Biotechnology & Biotechnological Equipment

ISSN: 1310-2818 (Print) 1314-3530 (Online) Journal homepage: https://www.tandfonline.com/loi/tbeq20

In Vitro Bulblet Induction from Bulb Scales of Endangered Ornamental Plant *Muscari Azureum*

S. Uranbey, A. Ipek, M. Caliskan, E. Dundar, S. Cocu, D. Basalma & H. Guneylioglu

To cite this article: S. Uranbey, A. Ipek, M. Caliskan, E. Dundar, S. Cocu, D. Basalma & H. Guneylioglu (2010) *In Vitro* Bulblet Induction from Bulb Scales of Endangered Ornamental Plant *Muscari Azureum*, Biotechnology & Biotechnological Equipment, 24:2, 1843-1848, DOI: 10.2478/V10133-010-0024-4

To link to this article: https://doi.org/10.2478/V10133-010-0024-4

ARTICLE DOI: 10.2478/V10133-010-0024-4 A&EB

IN VITRO BULBLET INDUCTION FROM BULB SCALES OF ENDANGERED ORNAMENTAL PLANT *MUSCARI AZUREUM*

S. Uranbey[1], A. Ipek[1], M. Caliskan[2], E. Dundar[3], S. Cocu[4], D. Basalma[5], H. Guneylioglu[2]
[1]University of Cankiri Karatekin, Faculty of Arts and Sciences, Department of Biology, Cankiri, Turkey
[2]Central Research Institute for Field Crops, Yenimahalle, Ankara, Turkey
[3]University of Balikesir, Faculty of Arts and Sciences, Department of Biology, Balikesir, Turkey
[4]University of Erciyes, Faculty of Agriculture, Department of Field Crops, Kayseri, Turkey
[5]University of Ankara, Faculty of Agriculture, Department of Field Crops, Turkey
Correspondence to: Serkan Uranbey
E-mail: emuranbey@yahoo.com, uranbey@karatekin.edu.tr

ABSTRACT

Muscari azureum with beautiful white and sky blue flowers is an important endangered ornamental plant of Turkey and needs exploitation for commercial propagation. 2-4 bulb scale explants of M. azureum were cultured in basal media supplemented with 2 mg/l 2,4-D, 20 g/l mannitol, 20 g/l sucrose, 0.5 mg/l NAA and different concentrations of BAP, KIN, 2iP and TDZ plus 2 g/l gelrite. The best regeneration on 2 or 4 scales and "the highest mean number of bulblets per explants (mean 8.77 per explant)" was achieved on an Orchimax medium supplemented with 2.0 mg/l BAP, 2 mg/l 2,4-D, 20 g/l mannitol, 20 g/l sucrose and 0.5 mg/l NAA for 2-scales. Mature bulblets were excised and individually rooted on half strength MS medium supplemented with 1 mg/l IBA, 0.5 g/l activated charcoal, 20 g/l sucrose and 6 g/l agar. Regenerated plants from 2 and 4 scales were acclimatized with a 14% survival rate after 3 weeks.

Biotechnol. & Biotechnol. Eq. 2010, **24**(2), 1843-1848

Keywords: *Muscari azureum*, bulblet, micropropagation, bulb scale

Abbreviations: MS: Murashige and Skoog medium; N_6: N_6 medium; BAP: N^6-benzylamino-purine; NAA: α-naphthaleneacetic acid; KIN: Kinetin; TDZ: Thidiazuron; 2iP: 2-isopentenyladenine; IAA: Indole-3-acetic acid; IBA: Indole-3-butyric acid; 2,4-D: 2,4-Dichlorophenoxyacetic acid

Introduction

M. azureum (syn. *Hyacinthus azureus*, *Hyacinthella azurea*) is commonly called "grape hyacinth" and is an important cultivated ornamental plant for pot and garden uses. The species is an herb with attractive flower colours and some selections are fragrant. It has sky-blue frilled bells with indigo stripes. The species flowers in medium spring (March/April) for 4-5 weeks and plants are usually 20-25 cm tall. The species is also included as an endemic and endangered species of Turkey and is 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 in future.

Plant micropropagation methods are powerful tools which provide means to vegetatively propagate plants. Also, plant tissue culture systems are very practical in virus elimination, rejuvenation of mature plant material and genetic transformation. Shoot regeneration via organogenesis and somatic embryogenesis has been reported for some of *Muscari* species (7, 10, 11, 12, 13, 16). However, shoot regeneration for *M. azureum* via either organogenesis or somatic embryogenesis has not been reported up to date. *In vitro* techniques may result in production of large number of bulblets in a short period. Therefore, *in vitro* multiplication of *M. azureum* can be valuable for commercial production and germplasm conservation. The aim of the study is also the development of a high frequency *in vitro* bulblet regeneration protocol for ornamental plant *M. azureum* using bulb scales.

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 (**Fig. 1a**) and planted in a Greenhouse. Bulbs were dried in the dark at room temperature for 2 weeks. After removing the roots and outer dry scales, the bulbs were washed in detergent. They were surface-sterilized by treatment for 3 min in 95% ethanol then in 100% commercial bleach for 40 min and finally rinsed five times with sterile water. Different sterile petri dishes were used in the isolation of bulb scales for each bulb.

Isolation of bulb scales

Bulb scale explants (3-5 mm width and 8-10 mm length) consisting of two and four scale segments attached to a thin segment of the basal plate were carefully isolated.

Culture conditions

Basal media salts, vitamins, sucrose, agar and growth regulators were obtained from Duchefa Biochem B.V. Netherlands. The pH of medium was adjusted to 5.7 with 1N NaOH or 1N HCl before autoclaving at 121ºC, 117.679 kPa for 20 min. All cultures were kept at 24±1ºC under cool white fluorescent light

(35 µmol m^{-2} s^{-1}) with 16-h photoperiod. All growth regulators (N^6-benzylamino-purine (BAP), Kinetin (KIN), Thidiazuron (TDZ), 2-isopentenyladenine (2iP), α-naphthaleneacetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D) were filter-sterilized using a Milipore filter (0.22 µm pore size) and added to hot autoclaved medium before dispensed into culture tubes. Bulb scale explants were cultured on 100-125 ml of bulblet induction media in Sterile Vent Container and subcultured several times on the same media. 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.

Bulb scale explants were cultured in the different bulblet induction medium as described below:

- *Bulblet induction medium* I: MS mineral salts and vitamins (8) + 2 mg/l 2,4-D, 20 g/l mannitol, 20 g/l sucrose, 0.5 mg/l NAA, different concentrations of BAP, Kinetin, 2iP and TDZ, 2 g/l gelrite;
- *Bulblet induction medium* II: N6 mineral salts and vitamins (1), + 2 mg/l 2,4-D, 20 g/l mannitol, 20 g/l sucrose, 0.5 mg/l NAA, different concentrations of BAP, Kinetin, 2iP and TDZ, 2 g/l gelrite;
- *Bulblet induction medium* III: Orchimax Medium mineral salts and vitamins + 2 mg/l 2,4-D, 20 g/l mannitol, 20 g/l sucrose, 0.5 mg/l NAA, different concentrations of BAP, Kinetin, 2iP and TDZ, 2 g/l gelrite.

Statistical analysis

5-10 bulb scale explants per Sterile Vent Container were used in the study for each replication. Each treatment had ten replicates and all experiments were repeated twice or more according to the contamination. 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 ($\sqrt{X}$) transformation (14) before statistical analysis.

Results and Discussion

Bulblet regeneration from bulb scales

Bacterial and fungal contaminations were observed on 5-10% explants which were eliminated. Bulb scale explants (3-5 mm width and 8-10 mm length) consisting of two and four scales were cultured in *bulblet induction medium* containing MS, N_6, Orchimax Medium basal medium supplemented with 2 mg/l 2,4-D, 20 g/l mannitol, 40 g/L sucrose, 2g/l gelrite and different concentrations of BAP, Kinetin, TDZ, 2-iP and NAA. All of 2 and 4 scales elongated, enlarged and formed green-yellow coloured compact callus about 3-4 weeks after culture initiation in all media tested. Embryogenic clusters and somatic embryos were visible after 10-12 weeks on compact calli. Shoot proliferation and bulblet formation were seen on

TABLE 1

Bulblet production from bulb scale consisting of 2 and 4-scale segments of M. azureum on bulblet induction medium I

Growth regulators (mg/l)						Explants producing shoots [%]		Mean number of bulblets per explant	
BAP	**KIN**	**2iP**	**TDZ**	**2,4-D**	**NAA**	**2 scales**	**4 scales**	**2 scales**	**4 scales**
0.25				2.0	0.5	0.0 e	0.0 e	0.0 e	0.0 c
0.50				2.0	0.5	27.5 b	0.0 e	**4.0 a**	0.0 c
1.0				2.0	0.5	5.0 d	17.5 b	0.5 cde	**3.5 a**
2.0				2.0	0.5	**37.5 a**	**32.5 a**	1.5 bc	1.75 b
	0.25			2.0	0.5	22.5 b	0.0 e	1.25 bcd	0.0 c
	0.50			2.0	0.5	2.5 d	0.0 e	0.25 de	0.0 c
	1.0			2.0	0.5	0.0 e	7.5 d	0.0 e	2.0 b
	2.0			2.0	0.5	10.0 c	27.5 a	1.25 bcd	**3.5 a**
		1.0		2.0	0.5	0.0 e	0.0 e	0.0 e	0.0 c
		2.5		2.0	0.5	0.0 e	0.0 e	0.0 e	0.0 c
		5.0		2.0	0.5	10.0 c	0.0 e	1.75 b	0.0 c
		10.0		2.0	0.5	0.0 e	15.0 bc	0.0 e	1.25 b
			00.5	2.0	0.5	0.0 e	0.0 e	0.0 e	0.0 c
			0.1	2.0	0.5	0.0 e	0.0 e	0.0 e	0.0 c
			0.25	2.0	0.5	0.0 e	0.0 e	0.0 e	0.0 c
			0.50	2.0	0.5	0.0 e	10.0 cd	0.0 e	1.0 bc

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

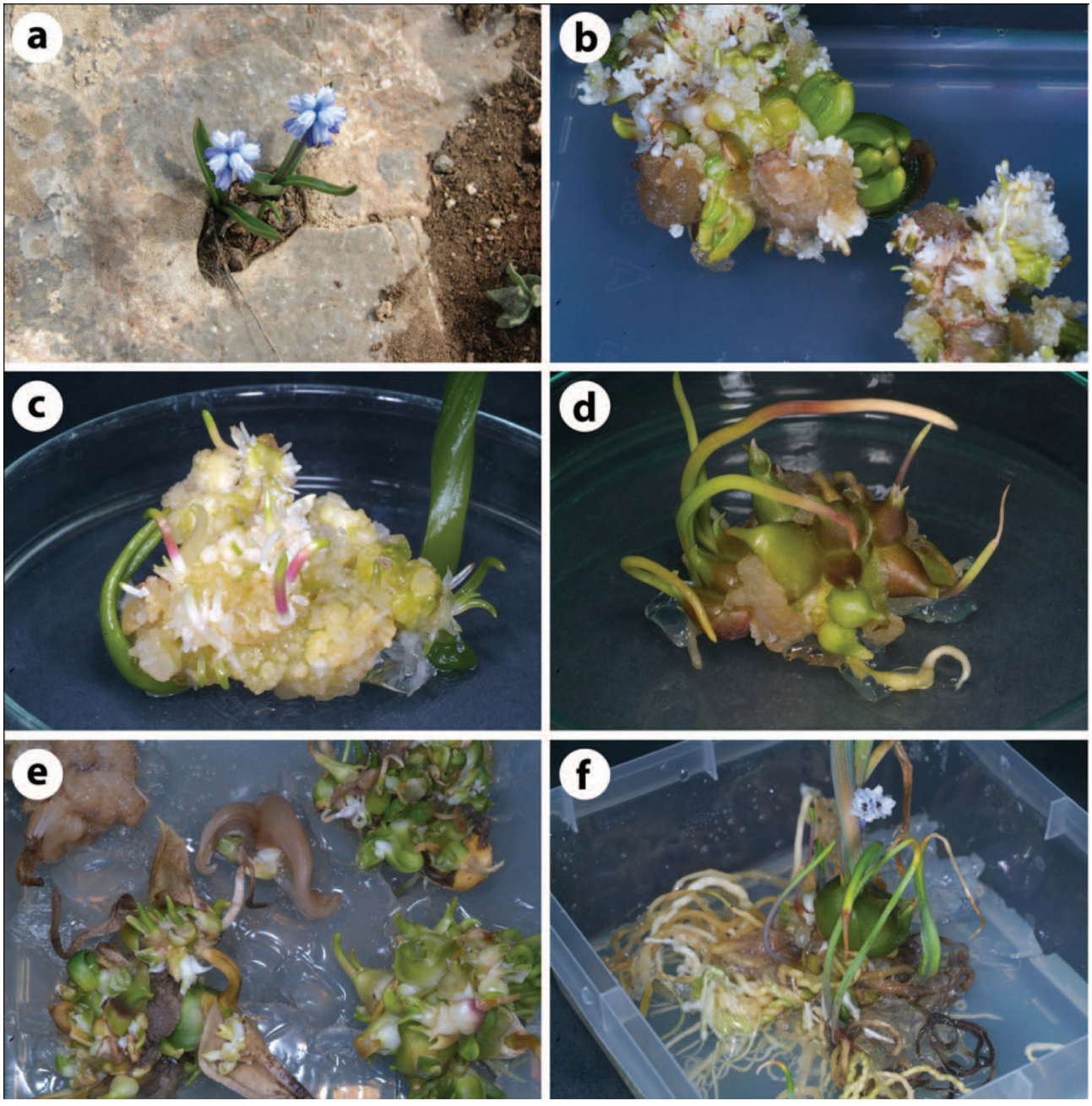

Fig. 1. Bulblet production 2-4 bulb scales of endangered species *M. azureum* (a) *M. azureum* growing in its natural habitat (b-c); green-yellow coloured compact callus formation and embryogenic clusters and somatic embryos on compact calli after 10-12 weeks in vulture (d-e); well developed bulblets on *bulblet maturation medium* after 6-8 months in culture (f); flowering of mature and rooted bulblet inside of Sterile Vent Container in growth chamber

2-4 scales 14-16 weeks after culture initiation (**Fig. 1b-c**). These shoots subsequently developed into small bulblets.

Effect of different growth regulators on bulblet production

The percentage of explants producing shoots and mean number of bulblets per explant were influenced on *bulblet induction medium* I ($p<0.01$). The highest percentage of regenerated shoots occurred on MS medium supplemented with 2 mg/l BAP for both two and four-scales. Whereas, the highest number of bulblets per explant was achieved on a MS medium supplemented with 0.50 mg/l BAP for 2-scales and was obtained on MS media containing 1 mg/l BAP and 2 mg/l KIN for 4-scales (**Table 1**).

Plant growth regulators were significant regarding the percentage of explants producing shoots and the mean number of bulblets per explant on *bulblet induction medium* II ($p<0.01$) for four-scales (**Table 2**). The maximum percentage of regenerated shoots (20.0%) occurred on N_6 media supplemented with 2 mg/l BAP and 10 mg/l 2-iP. Similarly, N_6 medium containing 2 mg/l BAP gave the highest mean number of bulblets per explant (2.77) for 4-scales.

TABLE 2

Bulblet production from bulb scale consisting of 2 and 4-scale segments of *M. azureum* on *bulblet induction medium* II

Growth regulators (mg/l)						Explants producing shoots [%]		Mean number of bulblets per explant	
BAP	**KIN**	**2iP**	**TDZ**	**2,4-D**	**NAA**	**2 scales**	**4 scales**	**2 scales**	**4 scales**
0.25				2.0	0.5	0.0	0.0 c*	0.0	0.0 c*
0.50				2.0	0.5	0.0	0.0 c	0.0	0.0 c
1.0				2.0	0.5	2.5	0.0 c	1.3	0.0 c
2.0				2.0	0.5	17.5	**20.0 a**	**2.75**	1.75 ab
	0.25			2.0	0.5	5.0	0.0 c	1.8	0.0 c
	0.50			2.0	0.5	2.5	0.0 c	1.32	0.0 c
	1.0			2.0	0.5	7.5	7.5 bc	1.05	1.3 bc
	2.0			2.0	0.5	2.5	0.0	0.32	0.0 c
		1.0		2.0	0.5	0.0	0.0 c	0.0	0.0 c
		2.5		2.0	0.5	2.5	0.0 c	0.0	0.0 c
		5.0		2.0	0.5	0.0	0.0 c	0.0	0.0 c
		10.0		2.0	0.5	0.0	**20.0 a**	0.0	**2.77 a**
			00.5	2.0	0.5	0.0	0.0 c	0.0	0.0 c
			0.1	2.0	0.5	0.0	0.0 c	0.0	0.0 c
			0.25	2.0	0.5	5.0	2.5 c	1.07	0.0 c
			0.50	2.0	0.5	7.5	0.0 c	0.0	0.0 c

*: 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

Bulblet production from bulb scale consisting of 2 and 4-scale segments of *M. azureum* on *Bulblet induction medium* III

Growth regulators (mg/l)						Explants producing shoots [%]		Mean number of bulblets per explant	
BAP	**KIN**	**2iP**	**TDZ**	**2,4-D**	**NAA**	**2 scales**	**4 scales**	**2 scales**	**4 scales**
0.25				2.0	0.5	0.0 b *	0.0 b**	0.0 b*	0.0 b*
0.50				2.0	0.5	0.0 b	7.50 ab	0.0 b	2.55 ab
1.0				2.0	0.5	10.0 ab	0.0 b	4.8 ab	0.0 b
2.0				2.0	0.5	0.0 b	0.0 b	0.0 b	0.0 b
	0.25			2.0	0.5	12.5 ab	12.50 ab	4.05 ab	0.0 b
	0.50			2.0	0.5	2.5 ab	0.0 b	1.57 b	0.0 b
	1.0			2.0	0.5	0.0 b	12.5 ab	0.0 b	**4.8 a**
	2.0			2.0	0.5	**15.0 a**	**20.0 a**	**8.77 a**	3.3 ab
		1.0		2.0	0.5	0.0 b	0.0 b	0.0 b	0.0 b
		2.5		2.0	0.5	7.5 ab	0.0 b	3.55 ab	0.0 b
		5.0		2.0	0.5	0.0 b	0.0 b	0.0 b	0.0 b
		10.0		2.0	0.5	0.0 b	0.0 b	0.0 b	0.0 b
			00.5	2.0	0.5	12.5 ab	7.5 ab	1.52 b	2.3 ab
			0.1	2.0	0.5	7.5 ab	0.0 b	3.77 ab	0.0 b
			0.25	2.0	0.5	0.0 b	0.0 b	1.57 b	0.0 b
			0.50	2.0	0.5	7.5 ab	0.0 b	4.55 ab	0.0 b

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

**: values within a column followed by different letters are significantly different at the 0.05 probability level using Duncan's multiple range test

The percentage of explants producing shoots and mean number of bulblets per explant showed that the frequency of shoot regeneration from different explants was influenced on *bulblet induction medium* III for 2-scales ($p<0.05$) (**Table 3**). Moreover, the influence of plant growth regulators was statistically significant at the level of 0.01 on the percentage of explants producing shoots for 4-scales, however, statistically important on the mean number of bulblets per explant at the level of 0.05 for 4-scales. The best percentage of explants producing shoots (15, 20%, respectively) was found on MS medium supplemented with 2 mg/l KIN for both 2 and 4-scales. The highest mean number of bulblets per explant (8.77) was achieved on an Orchimax medium supplemented with 2.0 mg/l BAP for 2-scales. However, Orchimax medium containing 1 mg/l BAP gave the best mean number of bulblets per explant (4.8) for 4-scales.

Shoots developed into small bulblets 4-6 months after culture initiation in all media tested and semi-developed bulblets were obtained 6-8 months after culture initiation (**Fig. 1d-e**). All bulblets forming on the different media were transferred to *bulblet maturation medium* containing MS mineral and vitamins, 20 g/l sucrose and 7 g/l agar. Bulblets were cultured on this medium for 3-4 months and they were subcultured every two weeks onto fresh medium. Bulbs elongated, enlarged and weighted on the maturation medium. Well-developed bulblets were separated and distributed to new culture vessels for further development. It was seen that some of bulblets bulb were with greenish brown colour skin on this medium. Mature bulblets were excised again and individually rooted on half 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 mature and rooted bulblets (approximately 10-15 mm in diameter) was achieved after 9-12 months in culture. It was seen that some of the mature bulbs flowered inside of Sterile Vent Container in growth chamber (**Fig. 1f**). Rooted bulblets were pre-treated at + 4°C for 4-6 weeks in dark before transferring to compost and peat mixture. Regenerated plants from 2 and 4 scales were acclimatized with a 14% survival rate after 3 weeks.

Taking both percentage of explants producing shoots and the mean number of bulblets per explant into account, it can be summarized that the highest bulblet regeneration capacity from two-scales was achieved on a Orchimax medium supplemented with 2.0 mg/l KIN. Of the various components of a tissue culture method, the basal nutrient medium is one of the most important factors influencing the success of culturing plant material *in vitro* (2). In the present study, substantial increases on number of bulblets per explant were achieved on media containing Orchimax mineral salts and vitamins when compared to other basal media. The type of explant is extremely important in establishing an efficient micropropagation and regeneration system (4, 18). Bulb scales are most commonly used as explants for in vitro micropropagation of geophytes. Earlier studies regarding shoot and bulblet multiplication in geophytes indicated that addition of growth regulators to basal media promoted bulblet regeneration from bulb scales of many geophytes (6, 9, 17, 19). BAP and KIN also generally increased the bulblet formation in our study. It can be said that KIN and BAP were more inductive for bulblet production than TDZ and 2-iP. In general, addition of TDZ and 2-iP to the media reduced the bulblet regeneration frequency as previously described in *Sternbergia fischeriana* (6). A superiority of BAP and KIN was reported in different explants of geophytes species (3, 5, 6, 15). The present study is in agreement with these studies and we also observed regeneration of healthy shoots in all on media containing BAP, KIN. Also, the media containing TDZ produced more vitrified and hollow bulblets.

Conclusions

M. azureum have low proliferation ratio and it takes three or four years to produce bublets in wild flora. Because of low propagation ratio, the commercial propagation of this species is limited. In the present study, we demonstrated the first report of *in vitro* bulblet production in endangered and endemic species *M. azureum*. The procedure described here may form the basis of germplasm conservation of *Muscari* species and commercial production of this important ornamental plant.

Acknowledgements

This study was supported by The Scientific and Technological Research Council of Turkey (TUBITAK, Project No: 106 O 034) We also thank to M.Sc. students and other friends for their assistance.

REFERENCES

1. **Chu C.C.** (1978) Science Press, 43-50.
2. **Gamborg O.L., Murashige T., Thorpe T.A., Vasil I.K.** (1976) In Vitro, **12**, 473-478.
3. **Ipek A., Cocu S., Uranbey S., Kaya D., Gurbuz B., Aslan N., Sancak C., Akdogan Ozcan S.** (2009) Research Journal of Biotechnology, **4**(4), 21-25.
4. **Koroch A., Juliani H.R., Kapteyn J., Simon J.E.** (2002) Plant Cell, Tissue and Organ Culture, **69**, 79-83.
5. **Malabadi R.B. and Van Staden J.** (2004) South African Journal of Botany, **70**(4), 618-621.
6. **Mirici S., Parmaksiz I., Ozcan S., Sancak C., Uranbey S., Sarihan E., Gumuscu A., Gurbuz B., Arslan N.** (2005) Plant Cell, Tissue and Organ Culture, **80**(3), 239-246.
7. **Mori S. and Nakano M.** (2004) Propagation of Ornamental Plants, **4**, 58-62.
8. **Murashige T. and Skoog F.** (1962) Physiologia Plantarum, **15**, 473-497.
9. **Paek K.Y. and Murthy H.N.** (2002) Plant Cell, Tissue and Organ Culture, **68**, 247-252.
10. **Peck D.E. and Cuming B.G.** (1986) Plant Cell, Tissue and Organ Culture, **6**, 9-14.

11. **Saniewski M. and Pytlewski C.** (1979) Bulletin De L' Academie Polonaise des Sciences, **27**, 519-521.
12. **Saniewski M. and Puchalski J.** (1987) Biologia Plantarum, **29**, 63-65.
13. **Saniewski M.** (1979) Bulletin dw L' Academei Polonaise des Sciences, **27**, 229-232.
14. **Snedecor G.W. and Cochran W.G.** (1967) Statistical methods, 6th Ed., The Iowa State University Press, Iowa, USA, p. 593.
15. **Suh J., Lee W., Lee A.** (2005) Acta Horticulturae, **683**, 155-163.
16. **Suzuki S. and Nakano M.** (2001) In vitro Cellular Development Biology, **37**, 382-387.
17. **Ulrich M.R., Davies F.T. Jr., Koh Y.C., Duray S.A., Egilla J.N.** (1999). Scientia Horticulturae, **82**, 95-102.
18. **Uranbey S., Cocu S., Sancak C., Parmaksiz I., Khawar K.M., Mirici S., Ozcan S.** (2003) Biotechnol. & Biotechnol. Eq., **17**, 33-37.
19. **Wawrosch C., Malia P.R., Kopp B.** (2001) Plant Cell Reports, **20**, 285-288.