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Research Article

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Bulblet Regeneration of Endemic *Muscari adilii* Güner & Duman on MS Media Having 6-Benzylaminopurine-α-Naphthaleneacetic

Abstract

Muscari adilii Güner & Duman is an endemic and threatened (CR) bulbous plant species, as is suggested by IUCN categorisation, therefore its export is prohibited. There is a need to protect this plant by developing *ex-vitro* and *in vitro* propagation techniques and protocols. Developing of these methodologies will conserve this species and prevent its extinction from nature. This study, aimed to develop a regeneration protocol for *M. adilii* on agar-hardened 1 × MS medium having different concentrations of benzylaminopurine (BAP)-α-naphthaleneacetic (NAA) using twin bulb scales and induced primary bulblets under *in vitro* conditions. The best medium for bulblet regeneration on twin scales was determined as 1 × MS medium having 17.76 µ Mol BAP- 2.685 or 10.74 µMol NAA (two combinations) obtaining 15.75 and 14.18 bulblets per explant in the same order. When the induced primary bulbs were used as explants, they regenerated 4.25 secondary bulbs on 1 × MS medium having 17.76 µMol BAP- 2.685 µMol NAA along with the increase in diameter of the induced primary bulbs. The maximum diameter of 0.39 cm of induced primary bulbs was noted on 1 × MS medium having 4.44 µMol BAP-5.37 or 10.74 µMol NAA. All of the bulbs were rooted in the regeneration medium, they were followed by successful transfer to the pots for acclimatization. The results of the study meets the aims of the study and it is possible to regenerate these bulbs for commercial propagation.

INTRODUCTION

All species in Genus Muscari Mill. - Asparagaceae family (Mulholland et al., 2013; Eroğlu, 2020) are important geophytes of commercial importance for their use in medicinal and ornamental plant industries (Şentürk and Binzet, 2021; Yıldırım, 2022) in Turkey. The bulbs of the Muscari species, after their flowering, continue their vitality underground until the start of the next-generation cycle (Polat, 2018). Bulbous plants contribute to the growth of the cut flower industry due to the high commercial demands, the possibility of dense planting, and higher yield per unit area (Chawla et al., 2020). Although no statistical data is available about the import-export or consumption of these plants in local markets (da Silva and Dobra'nszki, 2016), the researchers emphasize the production of Muscari species and their special place in the landscapes parks and garden decorations (Seyidoğlu, 2009; Onat, 2012; Kılıçarslan and Dönmez 2016). Endemic and non-endemic Muscari species in Turkey are classified according to the IUCN threat categories; first recognized and accepted in the 1980s (Ekim et al., 2000). This classification placed *M. adilii* local name "bey sümbülü" (Eker, 2012) is a critically endangered (CR) plant species which is at risk of extinction in near future (Ekim et al., 2000). The Ministry of Agriculture and Forestry publishes an export list of wildflower bulbs every year. All of the Muscari species are included in this list, and their export is prohibited and unlawful (Official Gazette, 2022). *M. adilii*, investigated in this study, grows at an altitude of 900-950 m at Ankara-Beypazarı and spreads in marl land which contains a carbonate-rich mud or mudstones with variable amounts of silt and clay. Flowering occurs in spring. The height of the plant is 4-15 cm. The light blue flowers are sterile and the dark blue-black colored flowers are fertile. (Güner and Duman, 1999). It is very important to develop propagation methods using conventional and *in vitro* techniques for the conservation of local germplasm to prevent their extinction (Bürün, 2021). Bulbous plants need 5-6 years from seed to blooming under ideal conditions in natural conditions (Yıldırım and Altun, 2021). A review of the literature shows *in vitro* multiplication reports of *M. armeniacum* (Bae et al., 2000; Suzuki and Nakano, 2001; Nakano vd. 2005), *M. comosum* var. *plumosum* (Xudong et al., 2006), *M. aucheri*, *M. azureum* (Uranbey, 2010a,b), *M. neglectum (*Karamian and Ranjbar, 2011; Roya et al., 2011), *M. mirum* (Nasırcılar et al., 2011.), *M. muscarimi* (Uzun et al., 2014), *M. aucheri* (Vaziri et al., 2014), *M. armeniacum* (Yücesan ve ark., 2014), *M. macrocarpum* (Ozel et al., 2007; Ozel et al., 2009), *M. muscarimi* (Ozel et al., 2015), *M. neglectum* (Ozel and Unal, 2016; Fida, 2020; 2021), and *M. racemosum* (Ozel and Unal, 2021) using aseptic cultures of *in vitro* induced bulblets, immature embryos, bulb scales, leaves, callus, and protoplasts explants. No studies have been found on the regeneration of *M. adilii* under *in vitro* conditions. Therefore, this study aimed to regenerate *M. adilii* using twin-scale explants and induced primary bulblets on 1 × MS medium having dissimilar concentrations and combinations of BAP-NAA under aseptic and *in vitro* conditions.

MATERIAL and METHODS

Plant material

M. adilii bulbs were obtained from the natural growing area of Doğandede Hills (Beypazarı Ankara). The diagnosis of bulbs was made at the Taxonomy section of the Department of Biology, Gazi University, Ankara, Türkiye.

Bulb sterilization and culture conditions

The *M. adilii* bulbs were held in the dark for two months. These bulbs separated into bulb scales were sterilized by keeping them in 1% v/v plant preservative mixture (PPM) for 120 minutes. The pH of each culture was set to 5.6-5.8 using 1 N Sodium Hydroxide (NaOH) or 1N Hydrochloric acid (HCl). Then, the respective cultures were sterilized in the Hirayama Hiclave Hv-

110 autoclave under a pressure of 1.4 kg/cm^2 at 121 °C for 20 minutes. All cultures having respective explants were placed and incubated in Sanyo versatile climate cabinets under white fluorescent light at 24 +/- 1°C under a day length of 16/8 hours' light and darkness. A total number of 16 explants were used in each treatment that was divided equally into four replications in regeneration and rooting experiments on 1 × MS (Murashige and Skoog, 1962) medium containing dissimilar concentrations of BAP and NAA in each treatment.

Statistical analyzes

The experimental data were analyzed with one-way ANOVA using "IBM SPSS 20 for Windows", Tukeys'b test was applied to separate means using the M-STAT C computer program. The values shown in percentage were arcsine transformed before subjecting them to variance analysis (Snedecor and Cochran, 1967).

RESULT and DISCUSSION

Bulb regeneration from bulb scales

The regeneration ability of twin scale explants of eight weeks stored bulbs was compared after sterilization on 1 × MS medium having dissimilar concentrations of BAP-NAA. The best regeneration was noted on 1 × MS medium having 17.76 µMol BAP and 2.685 and 5.37, or 10.74 µMol NAA. Karaoğlu (2010) emphasized that the bulbs kept in a dark and moisture-free place for 1-1.5 months after removal from the soil, became suitable for use in tissue culture since their enzymatic activities decreased. Similarly, Fida (2020) also approved these findings in *M. neglectum* bulb scales regenerated bulblet regeneration on bulb scales. The explants taken from fresh bulbs developed necrosis and died soon after. The researcher noted 100% regeneration on bulb scales taken from bulbs kept in the dark for 6 weeks. Similarly, Uranbey (2010 a,b), Ozel et al. (2007, 2009, 2015, 2021), Vaziri et al. (2014), Uzun et al. (2014), Özdemir (2017), and Fida (2020) investigated bulblet regeneration capacities using dissimilar auxin and cytokinin concentrations. At the end of eight weeks, bulblets were regenerated from the twin bulb scales of bulbs taken from wildlife. Nasircilar et al. (2011) used dissimilar explants of *M. mirum* and obtained the best results from bulb scale explants joined at the base. As seen in Table 1, the best medium for the number of bulbs per explant was 1 × MS medium having 17.76 µ Mol BAP- 2.685-10.74 µMol NAA. Consequently, 15.75 (Figure 1.a) and 14.18 (Figure 1.b) bulblets were induced in these cultures. The percentage of bulblet regeneration ranged between 22 and 100%. It was noted that the 1 × MS medium having 17.76 µ Mol BAP and 2.685 µMol NAA was the best medium inducing 100% bulblet regeneration. The diameters of the bulblets varied between 0.10 and 0.25 cm. The best medium for bulb buds induction per explant was 1 × MS medium having 17.76 µ Mol BAP and 10.74 µMol NAA with induction of 15.68 bulbs and 4.44 µMol BAP and 5.37 µMol NAA with induction of 15.00 bulbs. Similarly, Azad and Amin (2012) noted that *M. armeniacum* increased bulb bud induction in 1 × MS medium having 17.76 µM BAP + 10.74 µM NAA or 4.14 8.28 µMol IBA. The mean number of bulb buds per explant varied from 0 to 75%.

Table 1. Effects of dissimilar concentrations of BAP-NAA on regeneration of bulblets on twin-scale explants of *M. adilii*

Treatments BAP (µMol)	NAA (µMol)	Percentage of bulblet induction	Number of bulbs per Explant	Diameter of bulbs	Bulb buds induction (%)	Mean number of bulb buds per explant
4.44	2.685	100.00	5.33d	0.13	41.67	5.08ab
4.44	5.37	100.00	1.33f	0.19	66.67	15.00a
4.44	10.74	83.33	3.92e	0.15	0.00	0.00b
8.88	2.685	91.67	1.42f	0.10	41.67	5.08ab
8.88	5.37	75.00	1.25f	0.25	0.00	0.00b
8.88	10.74	91.67	7.83c	0.14	58.33	10.00ab
17.76	2.685	100.00	15.75a	0.10	41.67	8.42ab
17.76	5.37	100.00	11.00b	0.10	66.67	5.58ab
17.76	10.74	83.33	14.18a	0.22	75.00	15.68a
MS (Control)		22.00	2.67e	0.15	0.00	0.00b

* Means of the values in a single column followed by dissimilar letters are statistically diffrent as calculated by Tukey's test at $p<0.01$.

Induction of regeneration on induced primary bulblet explants

Twin-scale explants were used to induce primary bulbs as shown in the previous section. The induced primary bulbs on each treatment were removed from the explants and subcultured to increase their diameter and study their ability to induce 2ndary bulblets. At the end of 2.5 months, it was observed that the diameter of the induced primary bulbs increased and new secondary bulbs were induced on the periphery of the basal plate. The results are given in Table 2. The highest number of secondary bulblets was observed with 4.25 bulblets on 1 × MS medium having 17.76 µ Mol BAP and 2.685 µMol NAA (Figure 1.c). It was observed that the induced primary bulblets used as explants showed an increase in their diameters. 1 × MS medium having 4.44 µMol BAP-1 and 10.74 µMol NAA medium having MS, with the diameter increase 0.53-0.58 cm, respectively final diameter of induced primary bulblets (Figure 1.d-e). The greatest increase in diameter (0.39 cm) was in 1 × MS medium having 4.44 µMol BAP-1-10.74 µMol NAA. Similarly, Ozel et al. (2007) obtained the greatest increase in diameter in *M. macrocarpum* from the medium having 4.44 µMol BAP-1 µMol NAA. The results obtained by Fida (2020) also support our study. Fida (2020) obtained the largest bulblet diameter (0.97 cm) in 1 × MS medium having 4.44 µMol BAP + 0.17.76 µ Mol NAA in clonal bulb production from *M. neglectum*. Considering the number of secondary bulblets, it can be concluded that 1 × MS medium having 17.76 µ Mol BAP- 2.685 µMol NAA with a 4.25 mean number per induced primary bulblet is the best medium. The diameter of these bulblets is 0.12 cm. To increase the number of bulblets, high cytokinin (17.76 µ Mol BAP) gives positive results, while lower cytokinin (2 µMol BAP) is sufficient for an increase in bulblet diameter. Similarly, Ozel (2015) studied secondary bulblet production from *M. muscarimi* induced primary bulblet. 7.83 secondary bulblets were obtained in 1 × MS medium having 17.76 µ Mol BAP-10.74 µMol NAA. According to Mirici et al. (2005) *Sternbergia fischeriana* obtained a high rate of bulblet (80 mean number) regeneration from 1 × MS medium having 4 mg l^{-1} 6-benzylaminopurine (BA) and 0.25 mg l^{-1} α-naphthaleneacetic (NAA). Similarly, Faruq et al. (2019) obtained the most axillary bulbs from *M. armeniacum* bulbs in 1 × MS medium having high BAP (4.0 µM) and lower NAA (2.0 µM). Deswiniyanti and Lestari (2020) studied the regeneration of lily (*Lilium longiflorum*) bulbs.

Researchers stated that low concentrations of auxin (2.685 µMol NAA) or cytokinin (1µMol BAP) added to the medium accelerated the growth and development of lily micro bulblets.

Table 2. Effects of dissimilar concentrations of BAP-NAA on secondary bulblet regeneration from induced primary bulblets

Treatment BAP (µMol)	NAA (µMol)	The initial diameter of induced primary bulblets (cm)	The final diameter of induced primary bulblets (cm)	The difference in the induced primary bulblet diameters (cm)
4.44	2.685	0.13	0.23ab	0.20bc
4.44	5.37	0.19	0.58a	0.39a
4.44	10.74	0.15	0.53a	0.39a
8.88	2.685	0.10	0.31ab	0.21bc
8.88	5.37	0.25	0.35ab	0.10c
8.88	10.74	0.14	0.27ab	0.13c
17.76	2.685	0.10	0.34ab	0.24bc
17.76	5.37	0.10	0.33ab	0.23bc
17.76	10.74	0.21	0.45ab	0.24bc
MS (Control)		0.15	0.17b	0.02d
BAP (µMol)	NAA (µMol)	Secondary bulblet regeneration percentage	Mean number of secondary bulblets per induced primary bulblet	Secondary bulblet diameter (cm)
4.44	2.685	50.00ab	3.42ab	0.09
4.44	5.37	66.67ab	0.50c	0.08
4.44	10.74	58.58ab	2.50b	0.17
8.88	2.685	75.00ab	0.25c	0.13
8.88	5.37	66.67ab	1.75b	0.21
8.88	10.74	75.00ab	0.25ab	0.15
17.76	2.685	100.00a	4.25a	0.12
17.76	5.37	83.33a	1.00bc	0.12
17.76	10.74	75.00ab	0.50c	0.12
MS (Control)		0.00b	0.00d	0.00
Treatment BAP (µMol)	NAA (µMol)	Number of roots per explant (%)	Mean number of roots per explant	Mean root length (cm)
4.44	2.685	58.33	2.58ab	2.48b
4.44	5.37	66.67	2.75ab	0.80b
4.44	10.74	100.00	4.75a	1.14b
8.88	2.685	50.00	1.00 b	0.68b
8.88	5.37	83.33	2.58ab	1.31b
8.88	10.74	41.92	2.33ab	0.62b
17.76	2.685	41.67	1.42b	0.33b
17.76	5.37	75.00	2.17ab	1.14b
17.76	10.74	100.00	4.92a	0.76b
MS (Control)		100.00	1.83ab	10.85a

The differences between the means shown with dissimilar letters in the same column are significantly different at the 0.01 levels according to Tukey's b test.

Rooting and adaptation

All induced bulbs were rooted in the regenerated nutrient medium. The roots were thin and healthy. In terms of the number of roots, the best medium was 1 × MS medium having 17.76 µ Mol BAP-10.74 µMol NAA (4.92 roots per bulblets) and 17.76 µ Mol BAP- 2.685 µMol NAA (4.72 roots per bulblets). The longest roots were found in the control group (Figure 1.f). Due to the rooting of the bulblets in the regeneration medium, they were not rooted in any auxin-containing medium separately, and their adaptation was achieved by

transferring them directly to the pots (Figure 1.g). In this study, the lack of a need for a rooting medium may be due to the genotype of the plant or the type/dose of auxin added to the regeneration medium. Similarly, Peck and Cuming (1986) stated that 50% of the bulbs in *M. armeniacum* were rooted in the regeneration medium. Contrary to these studies, many researchers report rooting in auxin-having nutrient media. Kromer (1985, 1989) reported that the rooting of *M. racemosum* in 1 × MS medium having IAA was short and thick. Azad and Amin (2012) rooted *M. armeniacum* bulbs in 1 × MS medium having 0.5 - 4.0 µM IBA, NAA, or IAA. Radhika et al. (2020) tried to root *Dipcadi montanum* bulbs in 1 × MS medium having 2.685, -6.715 µMol NAA, 2.53, 5.07, 6.325 µMol IAA and 2. 45, 4.90, 9.80 µMol IBA. The maximum number of rooting with 15.2 roots per bulblet was reported on 1 × MS medium having 5.07 µMol IAA.

Figure 1. Regeneration, rooting and adaptation of *M. adilii* in 1 × MS medium having BAP-NAA (a) Regeneration in a medium having 17.76 µ Mol BAP- 2.685 µMol NAA (b) 17.76 µ Mol BAP-10.74 µMol NAA (c) Induced Primary bulblet regeneration on 1 × MS medium having 17.76 µ Mol BAP- 2.685 µMol NAA (d) Induced Primary bulblet regeneration on medium having 4.44 µMol BAP-1 µMol NAA (e) Induced primary bulblet regeneration on medium having 17.76 µMol L BAP-10.74 µMol NAA (f) Rooting on 1 × MS medium (g) Adaptation in pots

CONCLUSION

This research provides important information about the clonal propagation of *M. adilii* which has commercial importance. In the current study, regeneration of bulblets was accomplished by using bulb scales and induced primary bulblets as explants on 1 × MS medium having BAP-NAA as growth regulators. Rooting the bulblets in the regeneration medium without the need for a rooting medium is seen as an advantage and an important discovery. The current results provide a successful protocol for the clonal mass propagation of CR endemic *M. adilii.* The study meets the planned targets for the

conservation of Turkish germplasm for commercial production.

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