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STIMULATING EFFECTS OF DIFFERENT BASAL MEDIA AND CYTOKININE TYPES ON REGENERATION OF ENDEMIC AND ENDANGERED *MUSCARI AUCHERI*

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Abstract – In this study a simple protocol has been developed for the endemic and endangered *Muscari aucheri* plant. 2-4 bulb scale explants of *M. aucheri* were cultured in different basal media (Nitsch mineral salts and vitamins, Orchimax Medium mineral salts and vitamins) supplemented with 2 mgL^{-1} 2,4-D, 20 mgL^{-1} mannitol, 20 mgL^{-1} sucrose, 0.5 mgL^{-1} NAA and different concentrations of BAP, KIN, 2iP and TDZ. The medium was solidified with 2 mgL^{-1} Gelrite. The Orchimax and Nitsch & Nitsch medium promoted shoot and bulblet regeneration. BAP, KIN and 2-iP were suitable for induction of the bulblet for 2-4 bulb scales of *Maucheri*. The highest number of bulblets per explant for 2 and 4 scales was achieved on the Orchimax medium supplemented with 1 mgL^{-1} KIN and 2 mgL^{-1} KIN, respectively. Whereas, the maximum number of bulblets per explant for 4 scales was recorded on the Nitsch medium supplemented with 2 mgL^{-1} BAP. However, the Nitsch medium containing 10 mgL^{-1} 2-iP gave the best number of bulblets per explant for 2 scales. Bulblets acclimatized very well under greenhouse and garden conditions.

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, NAA - α-naphthaleneacetic acid; IBA - Indole-3-butyric acid; 2,4-D - 2,4-Dichlorophenoxyacetic acid

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INTRODUCTION

The genus *Muscari*, commonly known as grape hyacinth, is a group of deciduous plants native to Eurasia. *Muscari aucheri* is also an ornamental herbaceous plant of the genus Muscari and is native to Turkey. Its flowers are an attractive sky blue and the species blooms in spring. It is also an endemic and endangered species of Turkey, threatened by complete extinction. Irregular collection of the bulbs of *M. aucheri* from their habitat, erosion and the overgrazing of meadows of Anatolian, hamper the future cultivation of the species.

Plant tissue culture systems are very practical in virus elimination, rejuvenation of mature plant material, genetic transformation and *in vitro* rapid clonal propagation. Plant propagation via tissue culture is also a prevailing instrument for *ex situ* conservation of some endangered and endemic species as geophytes (Uranbey, 2006; Jevremoviç et al., 2009, İpek, 2009). *In vitro* bulblet production via organogenesis and somatic embryogenesis has been reported for some of the *Muscari* species (Saniewski and Pytlewski, 1979; Saniewski, 1979; Peck and Cuming, 1986; Saniewski and Puchalski, 1987; Suzuki and Nakano, 2001; Mori and Nakano, 2004, Uranbey et al., 2006). Although there are some reports on the regeneration of Muscari species, there are still no data concerning the micropropagation of *M. aucheri*. Therefore, this research was conducted to develop an efficient *in vitro* bulblet regeneration protocol as a commercial propagation and contribution to the preservation of this important endemic and endangered species.

MATERIALS AND METHODS

Plant material and surface sterilization

Bulbs of *M. aucheri* were collected from the wild flora of the Tokat and Ankara provinces of Turkey in April (Fig. 1a) and planted in a greenhouse. The 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 from each bulb.

Isolation of bulb scales

Bulb scale explants (3-5 mm in width and 8-10 mm in 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 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 a 16 h photoperiod. All growth regulators (*N^6-benzylamino-purine (BAP), Kinetin (KIN), Thidiazuron (TDZ), 2-isopentenyladenine (2-iP), α-naphthaleneacetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D*) were filter-sterilized using a Millipore filter (0.22 µm pore size) and added to hot autoclaved medium before being dispensed into culture tubes. Bulb scale explants were cultured on 100-125 ml of bulblet induction

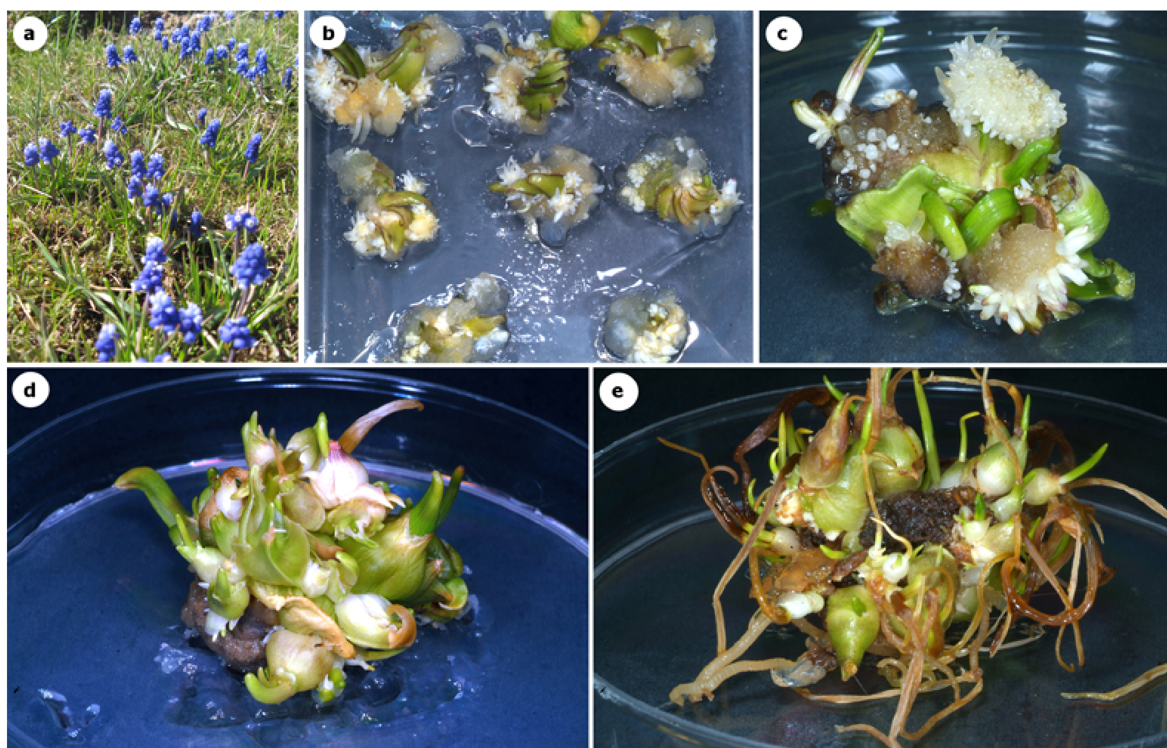

Fig 1. *In vitro* bulblet production 2-4 bulb scales of the endangered and endemic species *M.aucheri* (a) *M.aucheri* growing in its natural habitat (b) Embryogenic callus clusters and somatic embryos after 10-12 weeks on compact calli (c) Prolific bulblet formation on 4 scales 3-5 months after culture initiation (d-e) Well-developed bulblets obtained 7-8 months after culture initiation

media in a Sterile Vent Container and subcultured several times on the same media. All bulblets were subcultured and cultivated 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 below.

Bulblet induction medium I: Orchimax Medium mineral salts and vitamins + 2 mgL^{-1} 2,4-D, 20 gL^{-1} mannitol, 20 gL^{-1} sucrose, 0.5 mgL^{-1} NAA, different concentrations of BAP, Kinetin, 2-iP and TDZ plus, 2 g/l Gelrite.

Bulblet induction medium II: Nitsch Medium mineral salts and vitamins (Nitsch and Nitsch, 1969) + 2 mgL^{-1} 2,4-D, 20 gL^{-1} mannitol, 20 gL^{-1} sucrose, 0.5 mgL^{-1} NAA, different concentrations of BAP, Kinetin, 2iP and TDZ plus 2 gL^{-1} 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 at least 10 times according to the contamination. All data were pooled. Significance was determined by analysis of variance (ANOVA) and the differences between the means were compared by Duncan's multiple range tests using the MSTAT-C computer program (Michigan State University). Data given in percentages were subjected to arcsine ($\sqrt{X}$) transformation (Snedecor and Cochran, 1967) before statistical analysis.

RESULTS

2 and 4 scales of *M.aucheri* induced a yellow colored and large amount of compact calli about 6-7 week days after culture initiation in all media tested.

Embryogenic callus clusters and somatic embryos were visible after 10-12 weeks on the compact calli (Fig. 1b). Shoot proliferation and prolific bulblet formation were seen on 2-4 scales 3-5 months after culture initiation (Fig. 1c), well-developed bulblets were obtained 7-8 months after culture initiation (Fig. 1-e). Groups of these small bulblets were teased apart from the main tissue and transferred to new culture vessels for further development.

There was a statistical difference among the concentrations of cytokinine types and the percentage of explants producing shoots. The mean number of bulblets per explant influenced the *bulblet induction medium I* ($p < 0.01$). Among all the cytokinine concentrations and the concentrations applied, the highest regenerated shoot and bulblet ratio for 2 and 4 scales was found on the Orchimax medium containing 1 mgL^{-1} KIN (92.5 %) and 2 mgL^{-1} KIN (100 %), respectively. Similarly, the maximum number of bulblets per explant for 2 and 4 scales was also achieved on the Orchimax medium supplemented with 1 mgL^{-1} KIN (20.0) and 2 mgL^{-1} KIN (26.75), respectively (Table 1).

Bulblet induction medium II had a variable and statistically important effect on the frequency of bulblet regeneration for both 2-4 bulb scales ($p < 0.01$) (Table 2). The best percentage of explants producing shoots and bulblets (82.5 %) was found on Nitsch medium supplemented with 2 mgL^{-1} KIN for 2 scales. Also, the highest percentage of explants producing shoots and bulblets (95.0 %) was determined on Nitsch medium supplemented with 2 mgL^{-1} KIN and 2 mgL^{-1} BAP for 4 scales. Similarly, the maximum number of bulblets per explant (27.2) for 4 scales was recorded when the Nitsch medium was supplemented with 2 mgL^{-1} BAP. However, the Nitsch medium containing 10 mgL^{-1} 2-iP gave the best number of bulblets per explant (21.5) for 2 scales. An increase in the concentration of BAP, KIN and 2-iP had a positive effect on bulblet production.

The shoots developed on *Bulblet induction medium I* and *Bulblet induction medium II* transformed to small bulblets 4-6 months after culture initiation. Half-developed bulblets were observed 6-8 months after culture initiation. The

bulblets were transferred to *bulblet maturation medium* supplemented with MS mineral and vitamins, 20 gL^{-1}sucrose, 7 gL^{-1} agar. Bulblets were cultured on this medium for 3-4 months. Well-developed bulblets were separated and distributed to new culture vessels for further development. It was seen that some of the bulblets gave a greenish-brown colored skin on this medium.

DISCUSSION

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* (Gamborg et al. 1976). The results of the study showed that both Orchimax and Nitsch medium promoted shoot and bulblet regeneration. High bulblet regeneration was achieved on alternative plant tissue culture media such as Orchimax and Nitsch mineral salts and vitamins. Earlier studies regarding shoot and bulblet multiplication in geophytes indicated that the addition of growth regulators to the basal media promoted bulblet regeneration from the bulb scales of many geophytes (Ulrich et al., 1999; Wawrosch, 2001; Mirici et. al., 2005). BAP, KIN and 2-iP were suitable for the induction of bulblet production on 2-4 bulb scales of *M.aucheri*. Orchimax and Nitsch medium containing BAP and KIN was more potent in inducing bulblet regeneration compared to 2-iP. Moreover, TDZ did not effectively promote bulblet production compared to the three cytokinine types. A superiority of BAP and KIN was reported in different explants of the geophytic species (Malabadi and Van Staden, 2004; Mirici et al., 2005; Suh et. al., 2005; Ipek et al., 2009). The present study is in agreement with these studies. We also observed regeneration of healthy shoots in all of the media containing BAP and KIN. Also, the media containing TDZ produced more vitrified and hollow bulblets. Explant type is a critical factor in establishing efficient and reliable regeneration systems for geophytes and other species (Koroch et al., 2002; Uranbey et al., 2003; Uranbey 2005; Uranbey et al., 2005a; Uranbey et al., 2005b). In this study, it was also observed that 2-4 bulb scales had a potential in bulblet production and as a usable explant source.

The mature bulblets were excised again and individually rooted on a *rooting medium* containing ½ strength MS basal medium supplemented with 1 mgL^{-1} IBA, 0.5 mgL^{-1} activated charcoal, gL^{-1} sucrose and 6 gL^{-1} agar. It was not difficult to root the regenerated bulblets on the MS medium. Formation of mature and rooted bulblets (approximately 10-15 mm in diameter) was achieved after 12-14 months in culture. Rooted bulblets were pretreated at + 4°C for 4-6 weeks in dark before transference to a compost and peat mixture. Regenerated plants from 2 and 4 scales were acclimatized with a 25% survival rate after 3 weeks.

In conclusion, we describe a simple protocol for the endemic and endangered species *M.aucheri* by somatic embryogenesis. This protocol could be used for commercial production and *ex situ* conservation of this endemic species and plant improvement through *Agrobacterium*-mediated genetic transformation

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