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Efficient *in vitro* Microprapagation for the Conservation of Endemic and Endangered aucher-eloy grape hyacinth [*Muscari Aucheri* (boiss.) Baker]

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Abstract

Muscari aucheri (Boiss.) Baker family Liliaceae is endemic to Turkey and is threatened by complete extinction. To conserve the plant, in accordance with international agreements for the protection of endangered geophytes, collection of *M. aucheri* bulbs from the natural habitats is forbidden in Turkey. Due to poor diversity the natural propagation rate of the plant is relatively slow and it takes 4-5 years to develop a bulb of commercial size; which restricts large-scale cultivation of the plant. Development of conservation methods like *in vitro* propagation techniques, for conservation have low impact on wild populations as they use a minimum of plant material. The present study aimed to establish a strategy for efficient *in vitro* bulblet regeneration of this important geophyte using 4 scale bulb explants as an aid to breeding and multiplication of the plants. These explants were cultured on MS medium containing 0.5, 1.0, 2.0 mg/l BAP, 0.5, 1.0, 2.0 mg/l GA4 and 0.1, 0.2 mg/l IAA. The maximum number of bulblets per explant was induced on MS medium containing 0.5 mg/l BAP, 0.2 mg/l IAA. The bulblets regenerated on any variant of BAP-IAA and GA4-IAA were successfuly rooted on MS medium. About 17% of the rooted bulblets could be acclimatised. All acclimatized plants survived and showed flourishing growth under field conditions. Thus this protocol could be used in effective can multiplication and conservation of the plant under *in vitro* conditions.

Keywords: Bulblets, bulb scales, *in vitro*, micropropagation, *Muscari aucheri*

INTRODUCTION

The genus Muscari, *Muscari aucheri* (Boiss.) Baker commonly known as Aucher-Eloy grape hyacinth, is a group of deciduous plants endemic to Turkey.

It is an ornamental perenniel endemic herbaceous low risk (LR) threatened plant [2] It has sky blue attractive flowers; which, bloom in spring.

Unplanned collection of *M. aucheri* bulbs from wild habitat, greenhouse effects erosion, urbanisation and careless grazing of meadows has resulted in negative impacts on wild populations.

There is need to protect plant diversity and plant tissue culture can be successfully used for *ex situ* conservation of endangered and endemic species including geophytes [3].

In vitro bulblet production through somatic embryogenesis and organogenesis has been reported in some Muscari species [18], [16], [15], [17], [21], [7], [9], [22]and [13]. Under natural conditions, it takes 4-5 years for bulbs to grow and take desirable weight to reproduce and multiply.

This is very long and unpleasant period and which must be reduced to shorter period for rapid multiplication of the bulbs.

A possible alternative to this could be use of plant through plant tissue multiplication.

Therefore, this study aimed to develop a suitable protocol for reliable multiplication of *M. aucheri;* that will be helpful in both commercial propagation and conservation of this endemic plant.

MATERIALS AND METHODS

Plant Material and Surface Sterilization

Bulbs of *M. aucheri* were collected after identification by Prof. Dr. Neset Arslan of the Department of Field Crops, Ankara Univeristy, Ankara, from the Elmadag region of Ankara province of Turkey during April 2008. The bulbs were washed in commercial detergent for 30 min (Haci Şakir, Turkey) and dried using tissue papers and were stored in the dark at room temperature for 2 weeksin a well ventilated place.

Thereafter, removing the roots and outer dry scales, they were pretreated for 3 min in 95% ethanol, followed by sterilzation for 30, 40, 50, 60, 70, 80 and 90 min in 100 % commercial bleach (Ace, Turkey) for 50 min and 5 x 5 min rinsing with sterile bidistilled water.

Isolation of Explants

The bulbs were sliced longitudinally into 4 to obtain four scale explants. Each of the longitudinally bulb scales was 3-5 mm in width and 8-10 mm in length. The scales used in the experiment were attached by a thin segment at the basal plate.

Culture Conditions

Full strength MS (Murashige and Skoog 1962) basal media containing 0.5, 1.0, 2.0 mg/l BAP - 0.1, 0.2 mg/l IAA or 0.5, 1.0, 2.0 mg/l GA_4, 0.1, 0.2 mg/l IAA + 40 g/l mannitol were solidified with 8g/l agar in this experiment to obtain micropropagation. The pH of the medium was

adjusted to 5.6-5.8 with 1 N NaOH or 1 N HCl before autoclaving at 121ºC, 117.679 kPa for 20 min.

All cultures, were incubated in the dark at 24±1˚C under Philips-day light lamps TLD 36 W/54, Hungary (35 µmol m-2 s-1-) with 16 h light photoperiod for 4-6 weeks.

Thereafter, the bulb scale explants were cultured on 100 ml of bulblet induction media in sterile Vent Containers and subcultured every 4 weeks for 3 times. The micropropagated bublets were rooted on ½ strength MS macro & micro elements containing 1mg/l NAA supplemented with 500 mg/l Caesin hydrolysate and solidified with 6 gr/l agar for 4 weeks. The rooted bulblets (5-10 mm) were taken out from culture vessels; after removing their agar under tap water, before transfer to culture vessels incubated at 20-22°C in Sanyo versatile environmental test chamber, (Canada) using Philips-day light lamps TLD 36 W/54, (Hungary) with light intensity of 35 µmol m^{-2} s^{-1} and 16 h light photoperiod.

RESULTS AND DISCUSSION

Surface Sterilisation

The results showed that duration of sterilization period affected surface sterilization of the bulbs at significant level ($p<0.05$) and sharp differences were noted among treatment periods. It was noted that 30 and 40 minutes of sterilization was ineffective and could reduce contamination to 50% only. This contamination reduced further to 10-20% at 50, 60, 70 and 80 min. sterilization. The maximum sterilization (%10≥) was achieved at 90 min. All contaminated explants were eliminated in the autoclave to avoid undesired growth of fungus and bacteria.

Effects of Different Concentrations of BAP and IAA on Bulblet Regeneration

The explants began to swell after 21 days and developed callus after 28 to 30 days of culture in range of 40-93.3%. Shoot initials were recorded on almost all explants after 42-45 days of culture. These developed into green shoots after 55-60 days of culture leading to the developments of small bulblets after 80-90 days of culture.

The mean number of bulblets per explant varied in each bulblet induction medium. The results showed significant statistical differences ($p<0.01$) among different concentrations of BAP and IAA to induce bulblets. Frequency of bulblets regeneration ranged 33.3-93.3%. Number of bulblets per explant ranged 3.7 – 33.0 bublets per explant on MS medium containing 0.5 mg/l BAP-0.1 mg/l IAA and 0.5 mg/l BAP-0.2 mg/l IAA respectively. It was interesting to note that in each of three concentrations of BAP with IAA used in this experiment, any concentration of BAP with 0.2 mg/l IAA was better for induction of number of bulblets per explant compared to regeneration on any concentration of BAP with 0.1 mg/l IAA.

Mature bulbs were rooted on rooting media All bulblets showed profuse rooting. Rooted bulblets were transferred to culture vessels containing compost and incubated in growth chamber for acclimatisation.

Table 1. Effects of diffrent concentrations of BAP and IAA on frequency of callusing, bulblet regeneration and number of bulblets per explant

Effects of different concentrations of GA_4 and IAA on bulblet regeneration

MS medium containing different concentrations of GA_4 and IAA also had variable and significant also effects on callusing and bulblet regeneration of explants. These explants began to swell after 17-18 days of culture leading to induction of callus after 24 to 28 days in range of 60-100%. Frequency of bulblet regeneration ranged 53.3 to 93.3%. Number of bulblets per explant ranged 8.0-25.3. Maximum number of bulblets were recorded on 0.5 mg/l GA_4-0.2 mg/l IAA.

All bulblets on maturity were transferred on bulblets rooting media, where they developed profuse roots. Thereafter, these were acclimatised in culture vessels containing compost.

Availability of *in vitro* propagation technique is of particular importance for *M. aucheri*, since multiplication of the plant by seeds take 3 to 5 years to flowering. If the period from seed to bulb formation is reduced, it will help in reducing the time of maturing, flowering and seed production; which can also accelerate the rate of plant multiplication positively. This is desired in commercial multiplication of bulbous plants. A range of explants have been used for *in vitro* bulblet production from bulbous geophytes previously [11], [23], [6], [19], [4], [14]. The concentration of BAP-IAA and GA_4-IAA in the culture medium played a critical role for adventitious bulblet regeneration (Table 1, 2). This study showed that initiation and multiplication of adventitious bulblets was influenced by the concentration and combination of BAP with IAA and GA_4 IAA in contrast to [17] who found that the initiation of bulblets was strongly inhibited by auxins in *M. comosum* and *M. botryoides*.

The variants of BAP-IAA and GA_4-IAA had variable effect on the regeneration behavior of explants and induction adventitious bulblet regeneration. Type and concentration of plant growth regulators in the culture medium had significant influence on adventitious bulblet regeneration and induced variable number of bulbs per explant.

Table 1. Effects of various concentrations of BAP-IAA on bublet regeneration from four scale explants of *M. aucheri*

Plant growth regulators (mg/l)		Frequency of callusing (%)	Frequency of bulblets regeneration (%)	Number of bulblets per explant
BAP	**IAA**			
0.5	0.1	40.0 d	33.3 d	3.7 c
1.0	0.1	86.6 b	80.0 b	21.7 b
2.0	0.1	73.3 c	46.7	10.7 c
0.5	0.2	93.3a	93.3a	33.0 a
1.0	0.2	46.6 d	46.7 c	22.0 b
2.0	0.2	40.0 d	40.0 c	17.0 b

Values in a column followed by different letters are statistically different using Duncans Multiple Range Test ($p<0.05$)

Table 2. Effects of various concentrations of GA_4 and IAA on bublet regeneration from four scale explants of *M. aucheri*

Plant growth regulators (mg/l)		Frequency of callusing (%)	Frequency of bulblets regeneration(%)	Number of bulblets per explant
GA_4	**IAA**			
0. 5	0.1	60.0 c	60.0 c	8.0 e
1.0	0.1	100 a	93.3 a	15.1 c
2.0	0.1	73.3	73.3 b	11.7 d
0.5	0.2	100 a	93.3 a	25.3a
1.0	0.2	100 a	93.3 a	21.1 b
2.0	0.2	73.3 b	53.3 cd	9.3 e

Values in a column followed by different letters are statistically different using Duncans Multiple Range Test ($p<0.05$)

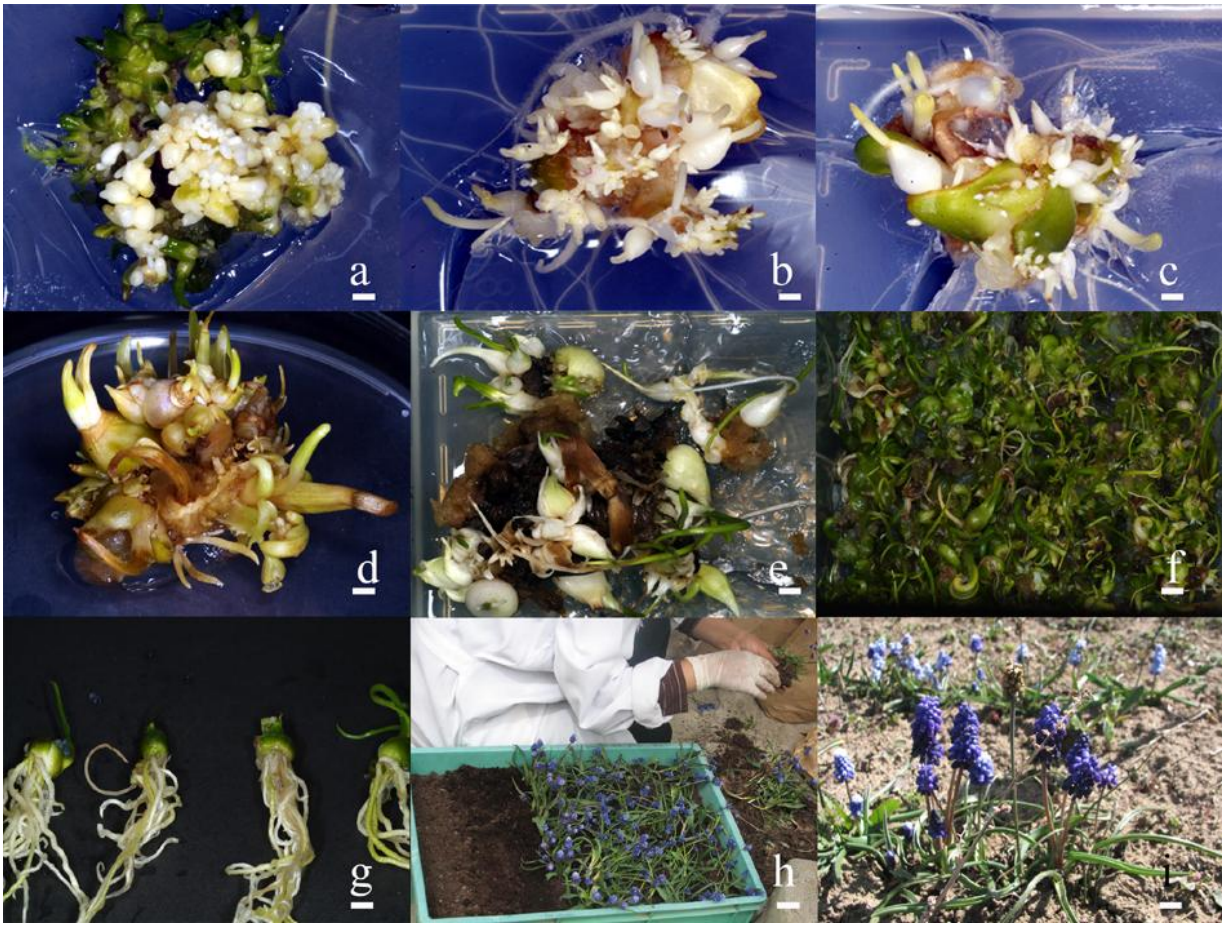

Fig 1. In vitro bulblet production of 2-4 bulb scales of the endangered and endemic species *M.aucheri* (a) *M.aucheri* growing in its natural habitat (b) Embryogenic callus clusters and somatic embryos after 10-12 weeks on compact calli (c) Prolific bulblet formation on 4 scales after 3-5 months of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

This study clearly shows that four scale explants of *M. aucheri* can be successfully cultured on BAP-IAA and GA_4-IAA containing medium to regenearate both bulblets. The results are in agreement with [1], who reported similar observations on bulb scale explants of *Urginea marititima.* It is well known that adventitous bulblet regeneration is undesirable in micropropagation because it can be source of somaclonal variation. However, particulary in case of *M.aucheri* it was not considered risky as the objective was conservation. No abnormality was recorded in the rooted and acclimatized bulblets.

The results are not compatible with [18], who observed bulblet regeneration from *M. Comosum* explants on MS medium without addition of growth regulators. The same authors empharesed that addition of NAA and adding of BAP inhibited bulblet formation in *M. comosum*.

Bulblets regenerated on BAP-IAA, GA_4-IAA combinations were easily rooted on ½ strength MS macro & micro elements, in agreement with the results of [12] with *Ornithogalum oligophyllum*. Our results also in agreement with [10], who found that bulblets of *O. umbellatum* could be rooted in half strength MS medium.

The paper meets the objectives of the study, i.e, the development of an effective and reproducible regeneration protocol for *M. aucheri* using four scale explants on MS medium, containing combinations of BAP -IAA and GA_4-IAA, it took 108 weeks only from initial culture to final acclimatization.

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