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ORIGINAL ARTICLE

Factors affecting efficient *in vitro* micropropagation of *Muscari muscarimi* Medikus using twin bulb scale

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Twin scale bulb explants

Abstract Endemic *Muscari muscarimi* Medikus is most fragrant plant among *Muscari* species and has high ornamental potential. The natural populations of *M. muscarimi*, is severely affected by increased environmental pollution and urbanization. There is need to develop a micropropagation method that should serve effectively for commercial propagation and conservation. Therefore, the study targeted to set up a strategy for efficient *in vitro* bulblet regeneration system of *M. muscarimi* using twin scale bulb explants on 1.0 × MS medium containing 4.44, 8.88, 17.76 μM BAP (6-Benzylaminopurine) plus 2.685, 5.37, 10.74 μM NAA (α-Naphthalene acetic acid). Maximum number of 19 daughter axillary bulblets and 16 daughter adventitious bulblets per twin bulb scale explant was regenerated on 1.0 × MS medium containing 17.76 μM BAP plus 10.74 μM NAA and 17.76 μM BAP plus 2.685 μM NAA respectively. The daughter bulblets regenerated on twin bulb scales on 8 out of 9 regeneration treatment could be easily rooted on 1.0 × MS medium containing 4.9 μM IBA (Indole-3-butyric acid). The daughter bulblets regenerated 9th treatment (1.0 × MS medium containing 17.76 μM BAP plus 10.74 μM NAA) were transferred to 1.0 × MS medium containing 30 g/l sucrose to break negative carry over effect of this dose of BAP–NAA, where they grew 2–3 roots of variable length. Daughter bulblet diameter was increased by culturing them on 1.0 × MS medium containing 4.44 μM BAP plus 5.37 μM NAA. The results verified that both age and the source of explants had significant effect on regeneration. In another set of experiments, twin scales were obtained from *in vitro* regenerated daughter bulblets, although they induced bulblets, yet their bulblet regeneration percentage, mean number of bulblets per explant and their diameter was significantly reduced. *In vitro* regenerated bulblets were acclimatized in growth

Abbreviations: MS medium, Murahige Skoog medium; BAP, 6-Benzylaminopurine; NAA, α-Naphthalene acetic acid; IBA, Indole-3-butyric acid.

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chamber under ambient conditions of temperature and humidity on peat moss, where they flowered. The study provides important information about selection of suitable micropropagation medium, strategies to improve bulblet diameter and rooting of *M. muscarimi* which offers a scope for commercial propagation.

1. Introduction

Unique location of Turkey lying between the temperate and subtropical regions (35–42 N and 25–45 E) have created a diversity of climates, habitats and ecosystems that has resulted in extraordinary plant diversity of 12,054 native vascular plant taxons on its soils. At least 3022 (33%) taxons among them are endemic, 2221 endangered and 584 as vulnerable or critically endangered (Hoekstra et al., 2005). Most of the vulnerable plant species belong to *Liliaceae*, *Amaryllidaceae* and *Iridaceae* families (Ekim, 2006; Tubives, 2013; Tehditaltındaki-Bitkiler, 2013).

Out of 25 *Muscari* species (family *Liliaceae*), found in Turkey (Davis, 1984; Cowley and Özhatay, 1994; Güner and Duman, 1999), ten species including ornamental *Muscari muscarimi* Medikus are endemic or vulnerable. *M. muscarimi* is most fragrant and scented among *Muscari* species and grows wildly in the Denizli and the Antalya provinces of Turkey (Ekim et al., 2000). It bears beautiful and attractive dirty gray–white flowers that bloom during June each year (Wraga and Placek, 2009; Tubives, 2013). A number of *Muscari* species are used as ornamental garden plants. However, commercial propagation of *M. muscarimi* as cut flower and outdoor garden plant has to be accomplished yet.

At present, due to number of abiotic and biotic stresses including increased pressure on natural resources, unsustainable agriculture and forestry practices, fast urbanization, increased industrial activities and fossil fuel consumption based increased CO_2 pollution (IPCC, 2010), *M. muscarimi* populations are under great pressure at its habitat. For *ex situ* conservation, the plant species has been included in the list of export prohibited plant species (Ekim et al., 2000).

No *ex vitro* or conventional *M. muscarimi* mass propagation system is available. The plants belonging to *Muscari* genus are multiplied by small offsets that originate around the basal plate of mother bulbs (Langeslag, 1989) and separated from them after lifting every 2nd year during July–August (Rudnicki and Nowak, 1993). Sometimes, it is preferred to obtain offsets by scooping or scoring the basal plate (Saniewski, 1975). Their seeds readily germinate; however, depending on suitable environmental conditions, the seeds induced plants take 4–5 years or even longer to mature, bloom and reproduce. Long and uncertain growth period of bulbous plants growth under natural conditions can be reduced through plant tissue culture techniques; which offer a possible alternative for rapid multiplication, conservation (Jevremovic et al., 2009) and commercial propagation.

A review of previous literature shows a few *in vitro* propagation studies in *M. comosum* (Saniewski and Pytlewski, 1979; Saniewski, 1979), *M. botryoides* (Saniewski and Puchalski, 1987), *M. armeniacum* (Suzuki and Nanako, 2001), *M. armeniacum*, *M. azureum*, *M. latifolium*, *M. moschatum*, *M. neglectum*, *M. paradoxum*, and *M. tubergenianum*, (Mori and Nakano, 2004), *M. macrocarpum* (Ozel et al., 2009), *M. aucheri* (Uranbey, 2010) and *M. mirum* (Nasircilar et al., 2010). Uzun et al. (2014) has recently reported regeneration of *M. muscarimi* using immature embryos as explant. There has been no report on *in vitro* propagation of *M. muscarimi* using twin scale explants and leaf.

Therefore, the study aimed to develop a reliable and fast *in vitro* regeneration system of *M. muscarimi* suitable for commercial propagation and unrestricted safe availability.

2. Material and methods

2.1. Plant material and surface sterilization

Clean dried 1.25–1.50 cm diameter bulbs of *M. muscarimi* that had been stored in shade and well ventilated place at room temperature (24 ± 2 °C) for 8 weeks were collected from the Department of Field Crops, Ankara University, Turkey. Their roots and outer scales were removed before surface sterilization using 80% (v/v) commercial bleach (Ace, Turkey, containing 5% NaOCl) and 1 ml/100 ml (v/v) of Tween 20 (surfactant) for 20 min. It was followed up by 5 × 3 min rinsing of bulbs with sterilized deionised water.

2.2. Isolation of explants regeneration and rooting

Depending on the bulb diameter, the mother bulbs were longitudinally sliced into 6 to obtain 0.3–0.5 cm wide and 0.8–1 cm long twin scale explants; attached by a thin segment at the basal plate to obtain 12 explants. Inner juvenile scales were not used in the regeneration experiments.

All explants were cultured on 1.0 × MS medium (Murashige and Skoog, 1962) containing 9 combinations of 4.44, 8,88, 17.76 μM BAP plus 2.685, 5.37, 10.74 μM NAA for 10 weeks of culture.

Rooting in all experiments was carried out using 1.0 × MS medium containing 4.9 μM IBA.

2.3. Culture conditions

The pH of the medium was adjusted to 5.7 ± 0.1 with 1 M NaOH or 1 M HCl before autoclaving at 121 °C, 117.679 kPa for 20 min.

All types of explants were incubated at 24 ± 1 °C under 16 h light (35 μmol $m^{-2}s^{-1}$) photoperiod provided by Philipsday–light lamps TLD 36W/54, Hungary.

2.4. Acclimatization

Healthy and well developed rooted *M. muscarimi* bulbs with shoot systems were acclimatized. The agar of the rooted

bulblets was removed after taking them out of Magenta culture vessels before transferring them to 1 l clay pots containing 0.75 l peat moss. Potting peat moss was locally prepared from leaves. It had pH 6.0 and electrical conductivity (EC) of 0.1 dS m^{-1}, with 67.5% (v/w) porosity, and allowed high water absorption with low bulk density of 0.1 mg m^{-3}. These pots were incubated at 24 ± 2 °C under 16 h light (35 μmol $m^{-2}s^{-1}$) photoperiod in growth room at 80% relative humidity and covered with transparent polythene bags to maintain a high relative humidity. Once the plants showed signs of growth, the polythene bags were pierced gradually to enable movement of gasses and adjustment of plants to external environmental conditions. Each pot was watered every day with 50 ml water during first week without flooding. Thereafter, the pots were watered, after every 3–4 days for 8 weeks. The plants hardened and showed signs of growth. The plants in all pots were uprooted carefully without damaging the root apparatus to note morphologic changes on bulb size and roots.

2.5. Statistical analysis

At the end of regeneration and rooting experiments, all experimental data was analyzed using one-way ANOVA of SPSS 16 statistical software. The post-hoc tests were performed using Tukey's b or Duncan's multiple range test with comparison made at 0.01 level of significance. Total number of 60 explants was used for each experimental treatment. The treatments were arranged in a completely randomized design. Each treatment was divided into 15 replicate groups containing four explants per replication.

3. Results

3.1. Bulblet regeneration on twin scale explants

The twin scale explants showed variable bulblet regeneration on 1.0 × MS medium containing 9 concentrations of BAP plus NAA used in the study. The bulb scales swelled after 7–8 days of culture followed by direct or indirect regeneration.

No callusing was recorded on MS medium containing 8.88 μM BAP plus 2.685 μM NAA, 17.76 μM BAP plus 10.74 μM NAA. A single axillary bulblet and a root was also noted on 1.0 × MS medium (Control). Rest of treatments had bulblets regeneration percentage in range of 41.67–100%. Furthermore, 100% bulblet regeneration was noted on 6 out of 10 treatments.

Number of bulblets per explant ranged 4.67–19.00 (Fig. 1a - Table 1) with bulblet diameter range of

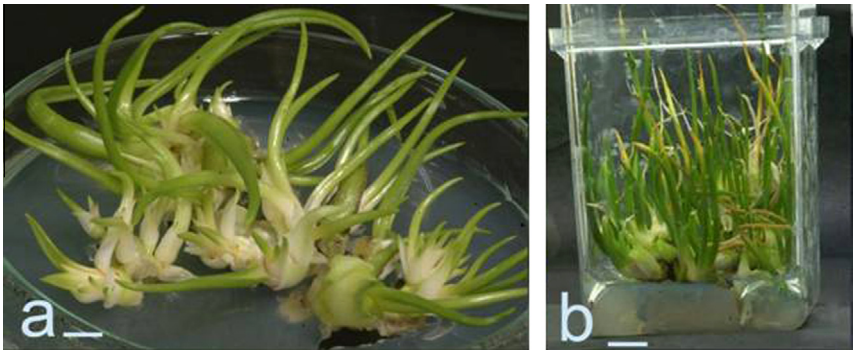

Figure 1 Micropropagation of *M. muscarimi* from twin mother bulb scale explants (a) daughter bulblet regeneration on 1.0 × MS medium containing 17.76 μM BAP plus 10.74 μM NAA after 10 weeks of culture on twin mother bulb scale explants (b) growing number of daughter bulblets induced on 17.76 μM BAP plus 10.74 μM NAA. Bar of (a) = 0.9 cm, (b) = 0.75 cm.

Table 1 Effect of different concentrations of BAP–NAA in MS medium on daughter bulblet regeneration from mother twin scale explants of *M. muscarimi* after 10 weeks of culture.

Plant growth regulators and their concentrations		Callus regeneration percentage (%)	Daughter Bulblet regeneration percentage (%)	Mean number of daughter bulblets per explant	Mean daughter bulblet diameter (cm)	Percentage of rooting (%)
BAP (μM)	NAA (μM)					
4.44	2.685	100.00a	100.00a	10.33d	0.40b	0.00b
4.44	5.37	100.00a	100.00a	8.00e	0.44a	0.00b
4.44	10.74	100.00a	66.67b	13.00c	0.28d	0.00b
8.88	2.685	0.00b	50.00b	5.33f	0.34c	0.00b
8.88	5.37	100.00a	41.67b	6.00f	0.41ab	0.00b
8.88	10.74	100.00a	66.67b	4.67f	0.36c	0.00b
17.76	2.685	100.00a	100.00a	16.00b	0.41ab	0.00b
17.76	5.37	100.000a	100.00a	14.00c	0.28d	0.00b
17.76	10.74	0.00b	100.00a	19.00a	0.36c	0.00b
MS (Control)		0.00b	100.00a	1.00 g	0.30d	100.00a

Means followed by different letters in same column are different using Tukey's b test at 0.01 level of significance.

0.28–0.44 cm, excluding Control (MS medium). Maximum number of daughter axillary bulblets was registered on 1.0 × MS medium containing 17.76 μM BAP plus 10.74 μM NAA. Maximum number of adventitious bulblets (16) were noted on 17.76 μM BAP plus 2.685 μM NAA.

All daughter bulblets were subcultured twice after eight weeks to increase diameter. Mean bulblet diameter varied in range of 0.90 –1.24 cm. Maximum number of 8 grand daughter bulblets were induced on 17.76 μM BAP plus 10.74 μM NAA irrespective of subculture (Fig. 1b). Maximum increase in daughter bulb diameter (1.24 cm) was noted on 4.44 μM BAP plus 5.37 μM NAA. The daughter bulb diameter could be compared with mother bulb diameter used to obtain explants (Values are not shown in tabulated form).

A number of twin scale explants on 6 out of 10 treatments induced 0.1 cm diameter daughter bulblets that were not counted at the time of taking final data. These bulblets were subcultured on 1.0 × MS medium containing 4.44 μM BAP plus 5.37 μM NAA to increase their bulblet diameter. A sharp increase in diameter of each daughter bulblet was noted that ranged 0.49–1.12 cm in 32 weeks time (Fig. 2a - Table 2). They also induced granddaughter bulblets in range of 16.67–100.00%. Their number and width changed 0.58–4.42 and 0.10–0.32 cm per explant, respectively. Each developed daughter bulblet had variable number of root initials that grew to roots of variable length vigorously on MS medium supplemented with 30 g/l sucrose (Fig. 2b – Rooting data not given in tabulated form).

The twin bulb scales obtained from *in vitro* regenerated daughter bulblets also showed variable but parallel response to regeneration on 1.0 × MS medium containing 9 concentrations of BAP plus NAA. However, their percentage of granddaughter bulblet regeneration, number of granddaughter bulblet per explants and granddaughter bulblets diameter were significantly less (Data are not shown in tabulated form).

3.2. Rooting

Preliminary experiments on rooting of largest bulblets obtained from 4.44 μM BAP to 5.37 μM NAA (as shown in Table 1) using different concentrations of NAA and IBA in 1.0 × MS and ½ × MS medium showed that best rooting was possible using 4.9 μM IBA. Therefore, 4.9 μM IBA was preferred in rooting of the bulbs obtained on 9 different regeneration media (as given in Table 1) at the final stage of the experiment. The rooting started on 1.0 × MS medium containing 4.9 μM IBA after one week of culture on 7 out of 8 treatments. However, carry over effect of variable concentrations of BAP–NAA in mother regeneration treatments affected their rooting variably. As the bulblets regenerated on MS medium developed single roots, therefore they were not rooted separately.

Excluding, non rooting on 17.76 μM BAP plus 10.74 μM NAA, the bulblets regenerated on different regeneration media had rooting percentage range of 33.33–100%. Their average number of roots per explant varied 0.42 to 5.25 per rooted

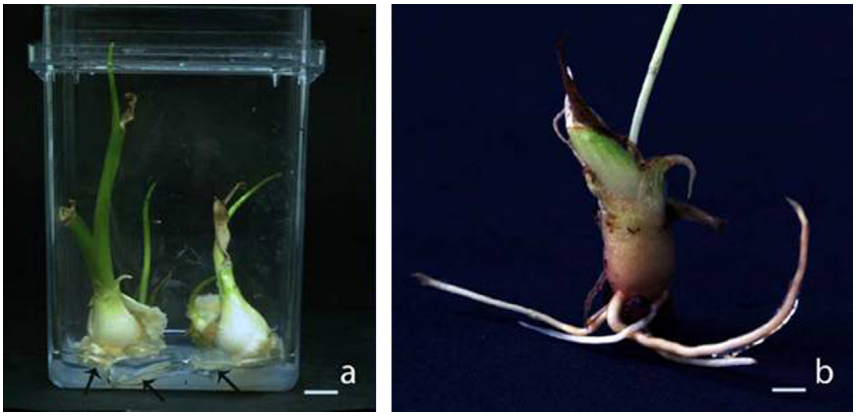

Figure 2 Increasing daughter bulblet diameter (a) an increased daughter bulblet diameter noted after 32 weeks of culture of 0.1 cm diameter daughter bulblets (b) daughter bulblets rooted on 1.0 × MS medium. Bar of (a) = 1 cm, (b) = 0.5 cm.

Table 2 Increasing daughter bulblet diameter of *in vitro* regenerated 0.1 cm diametered daughter bulblets from various cultures on MS medium containing 4.44 μM BAP–5.37 μM NAA.

Plant growth regulators and their concentrations		Final daughter bulblet diameter (cm)	Percentage (%) of daughter bulblets	Mean number of daughter bulblets per explant	Mean diameter of daughter bulblets
BAP (μM)	NAA (μM)				
4.44	2.685	0.49c	91.67ab	3.92a	0.13b
4.44	5.37	0.64bc	33.33c	0.92bc	0.11b
4.44	10.74	0.64bc	50.00bc	2.67ab	0.10b
8.88	5.37	1.12a	25.00c	1.12bc	0.10b
17.76	5.37	0.89ab	16.67c	0.58c	0.10b
17.76	10.74	0.79abc	100.00a	4.42a	0.32a

Means followed by different letters in same column are different using Duncan's multiple range test at 0.01 level of significance.

daughter bulblets and attained root length range of 0.13–8.00 cm. Maximum rooting percentage, number of roots per explant and their length was noted on larger bulblets induced on 4.44 μM BAP plus 5.37 μM NAA (Values not shown in tabulated form).

The axillary daughter bulblets induced on best treatment (17.76 μM BAP plus 10.74 μM NAA reported above) was suppressive for rooting but induced variable number of axillary granddaughter bulblets instead. These, when cultured on 1.0 × MS medium plus 30 g/l sucrose induced 100% roots after four weeks with mean number of 1.60 roots per daughter bulb and root length of 3.57 cm (Values are not shown in tabulated form).

Table 3 Variations in acclimatization of daughter bulblets regenerated on different concentration of BAP–NAA rooted on 4.9 μM IBA.

Plant growth regulators and their concentrations used in regeneration of daughter bulblets		Mean number of roots per explants	Mean root length (cm)
BAP (μM)	NAA (μM)		
4.44	2.685	1.33bc	6.42
4.44	5.37	4.00a	1.67
4.44	10.74	1.33bc	11.70
8.88	2.685	3.33ab	3.69
8.88	5.37	2.00abc	3.55
8.88	10.74	1.00bc	1.50
17.76	2.685	1.33bc	13.40
17.76	5.37	2.00abc	11.44

Means followed by different letters in same column are different using Tukey's b test at 0.01 level of significance.

3.3. Acclimatization

Irrespective of the bulblet regeneration medium, all *in vitro* regenerated daughter bulblet roots were thick and brittle. The variably rooted daughter bulblets were transplanted to pots containing peat moss, and transferred to growth room for hardening and acclimatization. They were taken away from pots after two months. Visible changes in the acclimatized bulblets was noted. Transplantation of *M. muscarimi* altered the root system. *In vitro* regenerated roots were replaced by changing number (1.33–4) of new longer roots (1.67–13.40 cm) with number of lateral and sublateral branches that grew horizontally and downward to the bottom of the pots. The longest roots were noted on daughter bulblets regenerated on 17.76 μM BAP plus 2.685 μM NAA (Table 3). The result of other experiment showed more than 80% acclimatization success of rooted plants. It was closely followed by 11.44 roots on 17.76 μM BAP plus 5.37 μM NAA (Fig. 3a). All mature acclimatized plants induced flower buds and bloomed (Fig. 3b, c) with induction of seeds. The plants were morphologically similar to the plants that grow under natural conditions.

4. Discussion

Plant tissue culture techniques provide possibility to introduce new approaches for direct regeneration depending on strong

Figure 3 (a) Acclimatization of *M. muscarimi* (a) root regeneration noted on daughter bulblets regenerated on 17.76 μM BAP plus 2.685 μM NAA (b, c) flowering of acclimatized plants in pots and fields. Bar of (a) = 0.6 cm, (b) = 1.6 cm, (c) = 1 cm.

competence of the genotype and *in vitro* culture conditions (Ochatt et al., 2013). The major problem to regeneration from any plant could be right choice of the explants and plant growth regulator combinations. *In vitro* micropropagation of *M. muscarimi* is very important and needs attention for development of micropropagation system for commercial use.

1.0 × MS medium supplemented with 9 variants of BAP plus NAA provided profuse and high percentage shoot regeneration on twin scale explant used in this study. 1.0 × MS medium containing 8.88 μM BAP plus 2.685 μM NAA and 17.76 μM BAP plus 10.74 μM NAA were highly suitable for direct organogenesis based axillary daughter bulblet induction. Rest of the 7 variants of BAP plus NAA used in the study induced callus based daughter adventitious bulblet regeneration. This study reports, regeneration of 19 bulblets on a single explant in 10 weeks time from 1/12 part of a single bulb without callusing. If we consider, 12 explants from a single bulb and regeneration of 19 bulblets per explant. It will induce 12 × 19 = 228 bulblets from a single bulb in 10 weeks time. This implies that this method is highly efficient compared to bulblet regeneration using immature embryos. This method is independent of season and regeneration could be carried out any time. This percentage and induced number of daughter bulblets per explant is noticeable Uzun et al. (2014) regenerated maximum number of callus induced 59 (daughter) bulblets per explant on MS medium containing 4 mg/l BAP and 0.5 mg/l NAA (17.76 μM BAP and 2.685 μM NAA) after 1 year of culture initiation after continuous subculturing. *In vitro* regeneration from immature embryos is very tedious, laborious and costly. However, bulb scale based micropropagation is very fast, independent of season and regeneration could be carried out at any time.

Percentage of granddaughter bulblet regeneration, number of granddaughter bulblet per explants and their diameter were significantly less on "*in vitro* regenerated daughter bulblets" compared to mother bulb twin scale explants. This showed that competence of regeneration was strongly related to the age of the explant. This is also in agreement with the results of Raju and Mann (1970) in *Echeveria elegans*, who proposed internal anatomy of explants at the time of isolation that affect regeneration. The observations also confirm that a period of competence development is needed for regeneration from the explants in agreement with McDaniel (1984) and Christianson and Warnick (1985). A serious problem of *in vitro* regenerated daughter bulblets is difficult to increase their diameter. The study showed that it was possible to increase bulblet diameter to 1.24 cm. This daughter bulblet diameter (1.24 cm) is partially comparable with the mother bulb diameter (1.25–1.50 cm) used in the study to obtain explants.

Still in another experiment, *in vitro* regenerated approximately 0.1 cm large daughter bulblets gained substantial mass of 1.12 cm on 4.44 μM BAP plus 5.37 μM NAA after 32 weeks of culture. These results are very positive and are in partial confirmation to the earlier findings of Marinangeli and Curvetto (1997) in *Lilium longiflorum* and their hybrids. The researchers used TA (traumatic acid) to increase the bulb weight to 60%.

The experiment made use of 4.9 μM IBA for rooting of the bulblets in agreement with Nayak et al. (1997), who rooted *in vitro* regenerated shoots of *Acampe praemorsa* Roxb. on 4.9 μM IBA.

The bulblets regenerated on 1.0 × MS medium containing 17.76 μM BAP plus 2 mg/l NAA showed positive increase in diameter and induced 100% roots when daughter bulblets were cultured on 1.0 × MS medium containing 30 g/l sucrose. Ozel and Khawar (2007) also observed rooting and increase in bulb diameter of *Ornithogalum oligophyllum* on 1.0 × MS medium.

Acclimatization and hardening of *M. muscarimi* is an important step as has been reported in many bulbous plants (Preece and Sutter, 1991; Paek and Murthy, 2002; Priyakumari and Sheela, 2005; Khawar et al., 2005). Major problems of tissue culture plants is that they experience a desiccation jolt just after transplantation (Ozel et al., 2008) that could be reduced to considerable extent by covering the transplanted material with polythene bags to that enable plants to survive outside culture vessels. These precautions helped easy establishment and recovery of transplanted material in a short time under growth room conditions.

Morphological analysis of the roots of acclimatized and hardened *M. muscarimi* bulblets after two months showed induction of number of lateral sub-lateral roots that could easily penetrate in peat moss in agreement with Ozel et al. (2009), who noted similar pattern of rooting in *M. macrocarpum*. More than > 80% rooted plants transferred to the greenhouse were successfully acclimatized in two weeks time. Peat moss acclimatized *M. muscarimi* bulblets easily absorb nutrients from soil favorably resulting in healthy and their fast maturity in pots and under field conditions, where all of them bloomed after six months.

5. Conclusion

The establishment of a successful regeneration and acclimatization protocol of *M. muscarimi* using twin bulb scale explants provides an opportunity for application of biotechnological tools to multiply the plant. The results are very meaningful and provide solid information relating commercial and horticultural propagation. The present investigation is a preliminary study. Extension of this study purposely may help in multiplication of this plant with unrestricted and safe availability of *M. muscarimi* throughout the year.

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