BİTLİS EREN ÜNİVERSİTESİ
FEN BİLİMLERİ DERGİSİ

ISSN: 2147-3129 / e-ISSN: 2147-3188

Article Type : Research Article
Received : April 9, 2025
Revised : June 25, 2025
Accepted : August 1, 2025
DOI : 10.17798/bitlisfen.1672984

Year : 2025
Volume : 14
Issue : 3
Pages : 1563-1580

MICROPROPAGATION OF *MUSCARI LATIFOLIUM*, AN ENDEMIC PLANT OF TÜRKİYE

Nesrin BOZHAN [1,*], Ahmet ONAY [1]

[1] *Dicle University, Department of Biology, Diyarbakır, Türkiye*

**** Corresponding Author:*** *n_bozhan@hotmail.com*

ABSTRACT

This study aimed to develop a seed-based *in vitro* propagation protocol for *Muscari latifolium* to ensure large-scale plant production while minimizing the impact on wild populations. Utilizing seeds as initial explants offers a sustainable and non-destructive alternative to bulb-based methods, promoting genetic diversity and supporting conservation efforts. Sterilization was optimized using 20% Sodium hypochlorite (NaOCl) for 30 minutes, achieving 100% decontamination and 100% germination. The most effective bulblet induction was observed on MS medium supplemented with 1.0 mg/l 6-Benzylaminopurine (BAP), yielding the maximum average bulblet number (21.06 per explant) and optimal bulblet length (0.71 cm), significantly outperforming other cytokinin treatments, including Kinetin (KIN). In contrast, the combination of 1.0 mg/l BAP and 1.0 mg/l α-Naphthalene Acetic Acid (NAA) also demonstrated substantial bulblet production, albeit at a lower average number (5.60 per explant), but with enhanced bulblet diameter (0.69 cm) and a notable rooting rate of 75%. While BAP alone proved superior for shoot multiplication and bulblet induction, adding NAA improved bulblet morphology and rooting efficiency, emphasizing the importance of cytokinin-auxin synergy. Rooting was most successful (100%) with 2 mg/l indole-3-butyric Acid (IBA), producing robust roots, while acclimatization achieved a 47% survival rate using a sterile peat-soil mixture. The developed protocol provides a reliable, non-destructive method for the ex situ conservation and horticultural production of *M. latifolium*, leveraging seeds to reduce the need for bulb collection. This approach holds promise for propagating other endangered geophytes with similar challenges.

Keywords: Bulbet formation, Micropropagation, *Muscari latifolium*, Tissue culture.

1 INTRODUCTION

Biodiversity conservation has become an urgent global priority, particularly for endemic and endangered plant species that play essential ecological roles and harbour unique genetic resources. The Mediterranean basin, recognized as a biodiversity hotspot, is home to numerous endemic species, many of which are under severe threat due to habitat destruction, urbanization, and overexploitation [1]. Türkiye, situated at the crossroads of Europe and Asia, possesses a rich floristic diversity, with over 12,000 native vascular plant taxa, approximately one-third of which are endemic. However, many of these species, particularly bulbous geophytes, are increasingly at risk [2]. Among these vulnerable taxa, *Muscari latifolium* John Kirk, commonly known as the broad-leaved grape hyacinth, is an endangered bulbous plant species endemic to south-western Türkiye. This species is distinguished by its unique blue-violet inflorescence and horticultural appeal, making it valuable in ornamental plant cultivation [3]. However, its natural populations are confined to montane pine forests at elevations of 1100-1800 m, where anthropogenic pressures such as deforestation, land-use change, and excessive harvesting have led to significant population declines [4], [5]. The increasing demand for this species as an ornamental plant, coupled with the limited natural regeneration capacity of its seeds and bulblets, has necessitated urgent conservation efforts [1].

Conventional conservation approaches such as seed banking and in situ preservation are often inadequate for *M. latifolium* due to its recalcitrant seed dormancy and slow natural propagation [5]. Consequently, ex-situ conservation through *in vitro* micropropagation has emerged as a viable alternative for the rapid multiplication of endangered plant species while preserving genetic integrity and reducing pressure on wild populations [6]. *In vitro* propagation not only enables the large-scale production of genetically stable, disease-free plants but also facilitates the establishment of germplasm banks and supports reintroduction initiatives [7], [2].

Several studies have successfully applied *in vitro* micropropagation techniques to various *Muscari* species. For instance, in a study by Uranbey [8], high-frequency bulb regeneration for the endemic and endangered ornamental plant *Muscari azureum* Fenzl was successfully achieved using immature embryos. A protocol for *in vitro* propagation of *Muscari mirum* Speta, an endangered geophyte, was developed by Nasırcılar et al. [9]. In this study, bulb scale, scape, leaf and immature embryo explants of *M. mirum* were cultured in different nutrient media and supplemented with different concentrations of plant growth regulators (BAP, NAA, TDZ, Picloram, 2.4-D). In the study conducted by Karamanian et al. [10] on *Muscari neglectum* Guss, somatic embryogenesis was induced from bulb explants cultured in Murashige and Skoog

(MS) medium containing no plant growth regulators or supplemented with different concentrations of 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BA). The best results were obtained in medium containing no plant growth regulators or with lower concentrations of NAA and BA and 30 g/ml sucrose.

Yücesan et al. [11], developed an effective *in vitro* regeneration system for *Muscari armeniacum* Leichtlin ex Baker in Linsmaier and Skoog basal medium (LS) supplemented with varying concentrations (0.5, 1.0 or 2.0 mg/l) of benzyladenine (BA) alone or in combination with 0.5 mg/l of NAA. In the study conducted by Vaziri et al. [12] on *Muscari aucheri* (Boiss.), which is endemic to Türkiye and is under threat of complete extinction, it was aimed to establish a strategy for effective in vitro bulb regeneration of this important geophyte by using 4-scale bulb explants to assist in the reproduction and propagation of the plants. In the study conducted by Ozdemir et al. [13], it was aimed to accelerate the proliferation by developing an effective *in vitro* bulb regeneration strategy for *M. aucheri*, whose natural populations are seriously affected by increasing environmental pollution, urbanization and grazing. *Muscari muscarimi* Medikus has been propagated using twin bulb scale explants, achieving high bulblet regeneration rates under optimized cytokinin and auxin conditions [2]. Similarly, *M. armeniacum* has been propagated via direct bulblet proliferation from bulb and leaf explants, highlighting the importance of plant growth regulator combinations for successful regeneration [14]. Additionally, *M. neglectum* has been investigated for its *in vitro* regeneration potential, demonstrating successful bulblet formation and acclimatization, thereby supporting both conservation and horticultural applications [15]. Despite these advancements, there is a paucity of literature specifically focusing on *M. latifolium*, and no dedicated regeneration protocol has been established for its efficiency *in vitro* propagation.

Utilizing seed-derived explants for *in vitro* propagation offers several distinct advantages over conventional bulb-scale methods. Seed-based propagation ensures higher genetic diversity, which is crucial for conservation programs and enhances the adaptability of reintroduced populations [14]. Additionally, seed explants are generally less susceptible to microbial contamination than bulb or leaf tissues, simplifying sterilization procedures and improving culture success rates [14]. Furthermore, seeds are more readily available and can be stored for extended periods, allowing for scalable conservation efforts without depleting natural populations [6]. Another crucial aspect of this study is the successful transition from seed-derived plantlets to bulblet formation, which is a relatively underexplored approach in *Muscari* micropropagation. Compared to direct bulblet regeneration from bulb-scale explants, seed-

based propagation requires overcoming additional challenges such as dormancy-breaking, optimizing germination conditions, and ensuring efficient bulblet formation in culture. The ability to induce bulblet formation from seed-derived plantlets is a significant achievement, as it enables the production of genetically diverse propagules that can be further multiplied through clonal propagation. Given the critical conservation status of *M. latifolium*, the aim of this study was to develop comprehensive micropropagation from seed-derived explants (axenic stems).

2 MATERIAL AND METHOD

2.1 Plant Material and Seed Collection

Mature seeds of *M. latifolium* were collected from natural populations in the broad-leaved forest understory of Ayazma Recreation Area, Bayramiç, Çanakkale in May 2017. The identification and seed collection of *M. latifolium* were carried out by Assoc. Prof. Dr. Hüseyin Eroglu (Van Yüzüncü Yıl University) and Prof. Dr. Mehmet Fidan (Siirt University). Seeds were carefully selected based on uniform size and maturity to ensure optimum viability for *in vitro* culture. Figure 1 presents the general appearance of the flowering *M. latifolium* in its natural habitat.



Figure 1. General appearance of M. latifolium during the flowering stage in its natural habitat.

2.2 Surface Sterilization of Seeds

A two-stage surface sterilization method was applied to initiate aseptic cultures. Seeds stored at room temperature of 25±2 ºC in a dark environment until the studies were started. Before surface sterilization, the seeds were washed under running tap water for 10 min. Then, they were immersed in 70% ethanol for 1 min and then washed with sterile distilled water at

least three times. A surface sterilization protocol was optimized to obtain maximum viable explants with minimum sterilant concentration. For this purpose, the seeds were shaken in sodium hypochlorite (NaOCl) solutions at different concentrations (10%, 20%, 30% and 40%) for 20 min. The seeds were then washed five times with sterile distilled water to remove the sterilant residues on their surfaces. The effect of sterilization time (10, 20, 30 and 40 min) on decontamination of seeds was determined using the sterilant percentage (20% NaOCl) that had the highest germination rate among different NaOCl concentrations tested. The efficiency of each sterilization process was evaluated according to contamination rates and seed viability three weeks after culturing.

2.3 Culture Medium and Growth Conditions

Surface sterilized seeds were lightly scratched with a scalpel tip to increase germination and cultured in hormone-free Murashige and Skoog (MS) medium [16] solidified with 6.4 g/L agar. The pH of the medium was adjusted to 5.8 using 1N NaOH and 1N HCl before autoclaving at 121°C and 1.2 atm for 20 min. The cultures were transferred to fresh medium every six weeks. The cultures were incubated in a growth chamber at 25±2°C with a 16-h photoperiod provided by cool-white fluorescent lamps (light intensity: 3000 lux). Each treatment consisted of 20 seeds and each experiment was repeated three times.

2.4 Shoot Induction and Bulblet Formation

After germination (approximately 15 weeks), in order to initiate bulblet development, in vitro shoots were subcultured onto MS medium supplemented with different concentrations (0.5 mg/l and 1.0 mg/l) of two different cytokinins BAP and KIN. All cultures were kept at 25±2°C with a 16-hour photoperiod. Shoot formation was evaluated after 20 weeks, while callus formation was tracked after 10 weeks. Primary bulblets were subcultured onto MS medium with 0.5 or 1.0 mg/l BAP or KIN to encourage bulblet enlargement after bulblet initiation. The effects of BAP and NAA at varying concentrations (1.0, 2.0, and 4.0 mg/l) on adventitious bulblet proliferation were examined in the following studies to further improve the methodology. Four to five axillary bulblets per treatment were removed along with the shoots and moved in duplicate to new culture medium. After 15 weeks, the growth and multiplication rates of the bulblets were assessed.

2.5 *In Vitro* Rooting and Acclimatization

In contrast to traditional micropropagation methods that necessitate distinct rooting media, the bulblets examined in this study demonstrated the ability to form roots spontaneously in cytokinin-enriched MS medium. The development of roots was assessed by evaluating root length, quantity, and the overall vigour of the plantlets. For the acclimatization process, the rooted bulblets were gently removed from the culture vessels, rinsed under running tap water to eliminate any remaining agar, and then transplanted into a sterilized peat and peat-soil mixture (1:1) based commercial growing medium. Micropropagated plantles were irrigated regularly, and were covered with a transparent plastic bag to ensure high humidity during the initial acclimatization stage. Humidity levels gradually decreased by making small perforations in the plastic cover each day, and the frequency of watering was reduced from daily to every 3-5 days. After a period of 6 weeks, the plastic coverings were taken off, and the plantlets were moved to greenhouse conditions for further hardening.

2.6 Data Collection and Statistical Analysis

To assess the effectiveness of the micropropagation protocol, several critical parameters were monitored at various growth stages: Axenic cultures; The rates of contaminated and decontaminated axenic cultures were reported three weeks after initiation of culture. Seed germination rate; These values were recorded 15 weeks after culturing. Callus induction and bulblet formation; These were assessed at 10 and 20 weeks after culturing, respectively. Rooting and acclimatization success; These were observed at 8 and 6 weeks after culturing, in sequence.

For the statistical analysis, each treatment comprised 15 to 20 explants, with three replicates for each experiment. The data were analyzed using One-Way Analysis of Variance (ANOVA), followed by the Duncan test for post hoc multiple comparisons at a significance level of $p < 0.05$. Statistical analyses were conducted using SPSS version 25.0. The data presented as proliferation percentages underwent Chi-square (χ^2) analysis.

3 RESULTS AND DISCUSSION

3.1 Surface Sterilization of Seeds and Axenic Germination

The success of *in vitro* propagation is highly dependent on effective surface sterilization, which prevents microbial contamination while maintaining explant or seed viability to a large extent. The choice of sterilant and exposure duration played a critical role in optimizing germination rates of *Muscari* seeds.

3.1.1 Effect of NaOCl Concentrations on Decontamination and Germination

After three weeks of culturing, a 100% contamination was observed in the control group (untreated seeds), demonstrating the necessity of surface sterilization in preventing microbial growth. Seeds treated with sodium hypochlorite (NaOCl) at various concentrations showed complete sterilization, with no contamination detected across all treatments. However, germination rates varied significantly depending on the NaOCl concentration. The highest germination rate (90%**)** was achieved with 20% NaOCl**,** whereas higher concentrations led to a decline in germination percentage (Table 1). The reduction at 30% and 40% NaOCl can be attributed to the cytotoxic effects of hypochlorite, which may damage seed tissues and reduce viability. These results confirm that 20% NaOCl is optimal for balancing sterilization efficacy and germination success.

Table 1. Effect of different NaOCl concentrations on surface sterilization and germination of M. latifolium seeds.

NaOCl Concentration (%)	Contaminated Seeds (%)*	Germinated Seeds (%)*
Control	100 b	0 d
10%	0 a	55 b
20%	0 a	90 a
30%	0 a	50 b
40%	0 a	20 c

**Data were subjected to Chi square (χ^2) analysis. Values represent the mean of 20 explants per treatment. Different letters indicate significant differences ($p < 0.05$).*

Sodium hypochlorite (NaOCl) is a surface sterilizing agent widely used in plant tissue culture studies [17], [18]. However, high concentrations and prolonged exposure can damage seed tissues and reduce germination efficiency. Therefore, optimizing sterilization conditions is of great importance, especially for rare and endangered species. Although specific studies on *Muscari* species are limited, studies on similar plant species provide important information about the effects of NaOCl [19], [20], [21]. Table 1 shows that each explant in the control group was infected and did not germinate. Infection was completely prevented and 55% germination rate was obtained at 10% NaOCl concentration. The highest germination rate was obtained with 20% NaOCl (90%), and it was observed that germination rates decreased at higher concentrations (30% and 40%). This situation shows that high NaOCl concentrations can have a toxic effect on seeds and negatively affect germination and 20% NaOCl is the optimal concentration and other concentrations provide lower germination compared to this value. For example, in a study on *in vitro* germination of *Lathyrus chrysanthus* seeds, it was observed that

3.75% NaOCl concentration was applied for 15 minutes and gave the best results, but 5% NaOCl concentration damaged the embryo and reduced the germination rate. This study shows that high NaOCl concentrations can damage seed tissues [19]. Similarly, in the sterilization of *Ficus religiosa* seeds, the highest germination rate (63.33%) was obtained in 5- and 10-min applications of 10% NaOCl concentration, while higher concentrations were observed to decrease the germination rate [20]. It has been reported that the application of 2% NaOCl solution at 20°C for 30 minutes in the surface sterilization of *Onobrychis viciifolia* seeds gave the best results, while higher concentrations and temperatures negatively affected the germination and regeneration capacity [21].

3.1.2 Effect of NaOCl Exposure Duration on Sterilization and Germination

Further optimization was carried out by varying exposure times using the 20% NaOCl concentration. All exposure durations resulted in complete sterilization, confirming the effectiveness of NaOCl treatment. However, germination rates increased significantly with longer exposure, reaching 100% germination at 30 and 40 minutes (Table 2). These results suggest that prolonged NaOCl exposure enhances sterilization without adversely affecting germination, a crucial factor for seed-based propagation strategies.

Table 2. Effect of 20% NaOCl exposure duration on sterilization and germination of Muscari latifolium seeds.

NaOCl Exposure Time (min)	Contaminated Seeds (%)*	Germinated Seeds (%)*
10	0 a	75 b
20	0 a	90 b
30	0 a	100 a
40	0 a	100 a

**Data were subjected to Chi square (χ^2) analysis. Values represent the mean of 20 explants per treatment. Different letters indicate significant differences ($p < 0.05$).*

These findings confirm that a 30-minute treatment with 20% NaOCl is optimal for balancing microbial decontamination and maximizing germination success in *M. latifolium*. In Table 2, it is seen that infection was completely prevented at all times of 20% NaOCl application and germination rates increased as the application time increased. Especially in 30 and 40-minute applications, 100% germination rate was obtained. These results show that both infections can be prevented and high germination rates can be achieved with appropriate NaOCl concentration and application time. The application time of NaOCl is also important in terms

of sterilization efficiency and seed viability. For example, a study on *Arabidopsis thaliana* seeds reported that treatments with 80% and 100% bleach concentrations for more than 10 minutes significantly reduced the germination rate and caused various growth defects in the seeds. This suggests that long-term NaOCl exposure may have negative effects on seed viability [22]. In a study conducted on buckwheat (*Polygonum convolvulus* L.) seeds, maximum germination was obtained as a result of 6-8 hours of treatment with 6% NaOCl. However, longer treatments resulted in poor germination and seed shattering [23]. In another study, the application of 20% NaOCl for different periods in the surface sterilization of terebinth (*Pistacia terebinthus* L.) seeds was examined and successful results were obtained with appropriate time and concentration combinations [24]. These findings indicate that the concentration and application time of NaOCl are critical to the germination success of seeds and their effectiveness in tissue culture studies. Therefore, it is necessary to determine the optimal conditions for the use of NaOCl, especially for the protection of endangered species such as *M. latifolium*. Low concentration and appropriate application times are important both to prevent contamination and to maintain the viability of seeds. Our results also align with the findings in other species, where excessive NaOCl exposure has been linked to tissue damage and reduced germination efficiency. Optimizing sterilization conditions is particularly important for *M. latifolium*, given its endangered status and the need to maximize viable seedlings for conservation purposes.

3.2 Shoot Induction and Bulblet Formation

3.2.1 Shoot Induction and Multiplication

The efficiency of shoot induction was significantly influenced by cytokinin type and concentration ($p < 0.05$). Among the tested treatments, the highest shoot proliferation was obtained on MS medium supplemented with 1.0 mg/l BAP, resulting in an average explant length of 0.42±0.02 cm and 26% callus induction (Table 3). Lower cytokinin concentrations led to reduced shoot proliferation, whereas higher concentrations promoted excessive callus formation, negatively impacting shoot elongation. These results indicate that BAP was more effective than KIN for shoot induction, consistent with findings in other *Muscari* species.

Table 3. Effect of different cytokinin types and concentrations on shoot development of M. latifolium.

PGR Type and Concentration (mg/l)	Average Shoot Length* (cm)	Average Explant Diameter * (cm)	Callus Induction (%)**
Control	0.41±0.06 a	0.15±0.04 a	26 a
0.5 BAP	0.37±0.03 bc	0.10±0.00 b	20 a
1.0 BAP	0.42±0.02 a	0.10±0.00 b	26 a
0.5 KIN	0.25±0.01 c	0.10±0.00 b	13 a
1.0 KIN	0.30±0.03 c	0.10±0.00 b	20 a

** Means followed by the different lowercase letter in the column of each explant source are significantly different at $p < 0.05$ according to the Post Hoc Multiple Comparisons Test.*
*** Data were subjected to Chi square (χ^2) analysis.*

The findings presented in Table 3 reveal significant differences in terms of average shoot length, explant diameter, and callus induction across different treatments. These results are consistent with similar studies in literature and provide insight into the role of cytokine types and concentrations in plant tissue culture. As shown in Table 3, the highest average shoot length (0.42±0.02 cm) was achieved with 1 mg/l BAP, whereas the lowest average shoot length (0.25±0.01 cm) was recorded with 0.5 mg/l KIN. The control group exhibited a comparable shoot length (0.41±0.06 cm), suggesting that the addition of low concentrations of BAP or KIN did not significantly inhibit shoot growth. This outcome is in line with the findings of previous studies, where lower cytokine concentrations either had no effect or limited effects on shoot elongation. In our study, 0.5 mg/l KIN produced a notably shorter shoot length compared to BAP treatments, suggesting that KIN's effectiveness in promoting shoot elongation may be limited at lower concentrations. The explant diameter followed a similar pattern to the shoot length. The control group exhibited the largest explant diameter (0.15±0.04 cm). The application of 1 mg/l BAP and 0.5 mg/l KIN resulted in a reduced explant diameter (0.10±0.00 cm), indicating that cytokinin treatments may not significantly increase explant expansion. Callus induction was assessed across different cytokinin treatments. The highest callus induction (26%) was observed in the control and 1 mg/l BAP treatments, whereas KIN-treated explants exhibited lower callus induction (13-20%). This result is consistent with previous research that has suggested BAP is more effective in inducing callus formation compared to KIN. On the other hand, KIN showed less effectiveness in inducing callus, which is consistent with the lower callus induction observed in our study.

3.2.2 Bulblet Induction and Multiplication

Bulblet formation is a key developmental stage in the micropropagation of *M. latifolium*, as successful bulblet development is essential for acclimatization and further multiplication. All the treatments tested produced bulblets on MS medium 20-week period following the initiation of the cultures from shoot explants of *M. latifolium* (Figure 2). The results presented in Table 4 showed that 1.0 mg/l BAP resulted in the highest number of newly formed bulblets per explant (21.06±2.96)**,** while 0.5 mg/l BAP (9,67±0,90) led to lowest bulblet induction rates. Average bulblet length ranged between 0,71±0,09 to 0,30±0,08 cm in 1 mg/l BAP and 0.5 mg/l KIN, respectively. The MS medium enriched with BAP resulted in a greater quantity and length of bulblets compared to the medium supplemented with KIN. The average diameter of the bulblets ranged from 0,12±0,02 to 0,04±0,01 cm (Table 4). The highest average bulblet diameter of 0,12±0,02 cm was observed in the MS medium supplemented with 1 mg/l of BAP. The MS medium supplemented with 1 mg/l BAP yielded a significantly higher quantity and length of bulblets in comparison to the medium enhanced with KIN.

Table 4. Effect of different cytokinin types and concentrations on bulblet induction of M. latifolium shoots.

PGR Type and Concentration (mg/l)	Average Bulblets Number*	Average Bulblet Length* (cm)	Average Bulblets Diameter** (cm)
Control	13.40±4.84 ab	0.36±0.12 b	0.04±0.01 b
0.5 BAP	9.67±0.90 b	0.49±0.06 ab	0.11±0.01 a
1.0 BAP	21.06±2.96 a	0.71±0.09 a	0.12±0.02 a
0.5 KIN	10.80±3.95 b	0.30±0.08 b	0.09±0.03 ab
1.0 KIN	10.46±2.75 b	0.66±0.10 a	0.11±0.02 a

**Means followed by the different lowercase letter in the column of each explant source are significantly different at $p < 0.05$ according to the Post Hoc Multiple Comparisons Test*
***Bulblet diameter equal to or greater than 0.50 cm was recorded for 6 treatments.*

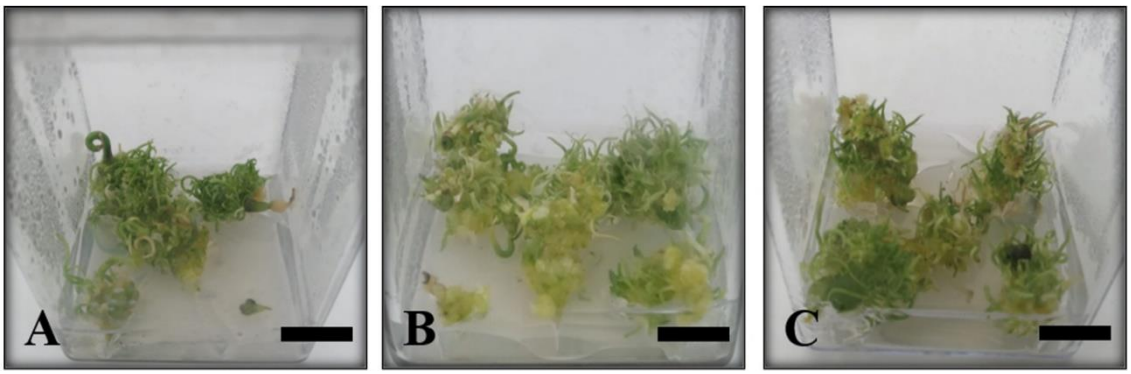


Figure 2. Development of M. latifolium bulblets in MS nutrient medium containing different PGRs after 20 weeks of culture period (A) Control medium (B) Bulblets in MS medium containing 1 mg/l BAP (C) Bulblets in MS medium containing 0.5 mg/l KIN. (Each bars: 1.2 cm).

As shown in Table 4, the highest bulblet number (21.06±2.96) was obtained with the application of 1.0 mg/l BAP. This result aligns with similar studies in the literature [9], [15], [25], which also indicate that BAP has a promotive effect on bulblet induction. In contrast, the control group yielded an average of 13.40±4.84 bulblets. Although this value is lower compared to the BAP treatments, it is still higher than the bulblet production in the KIN treatments. KIN applications, particularly at 0.5 and 1 mg/l concentrations, resulted in bulblet numbers ranging from 9.67±0.90 to 10.80±3.95 Regarding bulblet length, the highest average value was observed with 1 mg/l BAP treatment (0.71 cm). This finding is consistent with studies reporting the promotive effect of BAP on bulblet induction in other *Muscari* species, where optimal concentrations of BAP significantly enhanced bulblet number and size [9]. Additionally, KIN applications were also similarly effective, with 1.0 mg/l KIN yielding a bulblet length of 0.66±0.10 cm. This suggests that KIN may increase bulblet length at higher concentrations, although its effect could be limited at lower concentrations. In the control group, the bulblet length was observed to be 0.36±0.12 cm on average. In terms of bulblet diameter, 1 mg/l BAP again produced the highest value (0.12±0.02cm). In KIN treatments, the average bulblet diameter was observed to be 0.09±0.03 cm with 0.5 mg/l KIN, while it reached 0.11±0.02 cm with 1 mg/l KIN.

The data obtained in this study reveal that BAP is a more effective cytokinin for bulblet induction in *M. latifolium*. The 1 mg/l BAP treatment demonstrated the highest effect on bulblet number, length, and diameter. KIN, although effective at certain concentrations (particularly at 1 mg/l), showed more limited effects compared to BAP. In accordance with other studies in the literature, it can be concluded that BAP is a more effective growth regulator for bulblet induction in *Muscari* species [9], [15], [25].

3.2.2.1 Effect of BAP and NAA Combinations on Bulblet Development

The results presented in Table 5 show that the combination of 1.0 mg/l BAP + 1.0 mg/l NAA produced the highest number of bulblets (5.60±0.59) and the formation of callus was observed in all treatments tested; however, the highest rate and the values obtained for bulblet number, length and diameter in the other tested combinations were lower than the lowest rate obtained with only cytokinin (BAP or KIN) applications (see Table 4).

Table 5. Effect of different BAP and NAA combinations on bulblet development.

PGR Type and Concentration (mg/l)	Average Bulblets Number*	Average Bulblets Diameter* (cm)	Average Bulblets Length* (cm)	Rooting (%)**
1 BAP+1 NAA	5.60±0.59 a	0.19±0.02 a	0.69±0.06 a	75 c
1 BAP+2 NAA	2.67±0.54 bc	0.11±0.02 bc	0.30±0.05 b	80 c
1 BAP+4 NAA	1.27±0.33 c	0.06±0.01 c	0.14±0.04 b	100 a
2 BAP+1 NAA	2.20±0.41 bc	0.18±0.03 a	0.75±0.09 a	100 a
2 BAP+2 NAA	5.60±0.60 a	0.16±0.01 ab	0.59±0.04 a	100 a
2 BAP+4 NAA	3.20±0.39 b	0.21±0.03 a	0.68±0.08 a	90 b
4 BAP+1 NAA	2.80±0.81 bc	0.20±0.03 a	0.69±0.13 a	90 b
4 BAP+2 NAA	1.53±0.32 bc	0.19±0.03 a	0.81±0.16 a	100 a
4 BAP+4 NAA	1.73±0.59 bc	0.08±0.02 c	0.25±0.07 b	80 c

** Means followed by the different lowercase letters in the column of each explant source are significantly different at $p < 0.05$ according to the Post Hoc Multiple Comparisons Test*
*** Data were subjected to Chi square (χ^2) analysis*

In the process of *in vitro* bulblet production from axenic shoots of *M. latifolium*, two distinct concentrations of BAP and KIN (0.5 and 1 mg/l) were evaluated individually, along with a combination of three varying concentrations of BAP (1, 2, and 4 mg/l) paired with three different concentrations of NAA (1, 2 and 4 mg/l), including a control group (Table 4 and Table 5). The study successfully demonstrated a significant and abundant rate of bulblet regeneration from axenic stem explants cultured on MS medium supplemented with 1 mg/l BAP. The combination of BAP with NAA reduced bulblet regeneration and development (Table 5). These findings suggest that utilizing stem explants for the micropropagation of *M. latifolium* holds considerable potential. Our study clearly demonstrated significant and abundant bulblet regeneration rates from axenic stem explants cultured in MS medium supplemented with 1 mg/l BAP. In contrast, previous studies on various bulbous plants Malabadi and Van Staden; [26], Suh et al., [27] suggested that media containing TDZ improved regeneration potential. Uzun et al. [28], previously reported that dozens of bulblets per explant via callus could be produced, but this success was achieved after further subculturing 12–14-month period. However, in our study, bulblets were produced in a shorter timeframe (within 10 months of culture initiation). Although direct comparisons of bulblet numbers are difficult due to methodological differences, our findings regarding efficient callus and bulblet production are consistent with the results reported in *M. armeniacum* and *M. muscarimi* studies [11], [28], [2]. The efficiency of our protocol is further evidenced by the quality of the regenerated plantlets.

3.3 Rooting and Acclimatization

The cultured bulblets exhibited various rooting responses according to the IBA treatments applied (Table 6). Among the treatments tested, the highest rooting percentage (100%) was achieved on MS medium supplemented with 2.0 mg/l IBA**,** producing well-formed roots with an average root number of 3.33±0.39 (Table 6; Figure 3A). The percentage of root forming bulblet was decreased with IBA concentration of 1mg/l and the control group. Acclimatization is very important for the future ex vitro plant development. Acclimatization is possible by decreasing the relative humidity from 90% to 50% in a 6 week-period when a mixture of peat-based medium for hardening of the plantlets (Table 7; Figure 3B). Acclimatization trials revealed that transferring bulblets to a peat-soil mixture resulted in a 47% survival rate, significantly higher than using sterile peat alone (Table 7; Figure 3C).

Table 6. Effect of auxin concentrations on bulblet rooting of M. latifolium.

Auxin Concentrations (mg/l)	Rooting (%)**	Average Root Number*	Average Root Length* (cm)	Average Bulblets Number*
Control	90	3.40±0.29 a	1.25±0.16 b	0.53±0.35 b
1 IBA	75	2.53±0.32 b	1.65±0.25 a	2.33±0.77 a
2 IBA	100	3.33±0.39 a	1.94±0.30 a	3.00±1.00 a

**Means followed by the different lowercase letters in the column of each explant source are significantly different at $p < 0.05$ according to the Post Hoc Multiple Comparisons Test.*
*** Data were subjected to Chi square (χ^2) analysis*

Table 7. Effect of different composts on bulblet acclimatization of M. latifolium.

Compost Types	Difference of Average Bulblets Length* cm)	Viable Bulblets (%)**
Peat (Sterile)	0.60±0,30 a	20 b
Peat+Soil (Sterile)	1.08±0,34 a	47 a

**Means followed by the different lowercase letters in the column of each explant source are significantly different at $p < 0.05$ according to the Post Hoc Multiple Comparisons Test.*
***Data were subjected to Chi square (χ^2) analysis.*

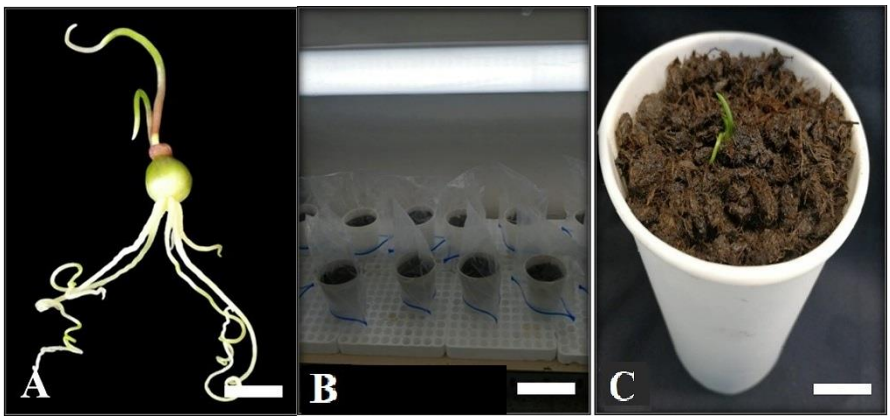


Figure 3. Rooting and acclimatization process of in vitro propagated M. latifolium: (A) Rooted bulblets in hormone-free MS medium (Bar: 1 cm); (B) Plantlets transferred to the plant growth chamber (Bar: 9 cm); (C) Hardened bulblets ready to transfer into Peat+Soil based compost (bar: 2 cm).

We observed that the *in vitro* bulblets rooted readily under an auxin-supplemented medium. Faruq *et al.* [14] showed that using a moderate auxin (2.0 µM IBA) in half-strength MS medium yielded well-formed roots in *M. armeniacum*, whereas higher auxin levels caused callus formation at the base of shoots. We adopted a similar approach, resulting in nearly 100% rooting of *M. latifolium* bulblets *in vitro*. In *M. neglectum* (grape hyacinth), Fida [29] likewise reported 100% rooting within 3 weeks on half-strength MS containing 0.5 mg/l NAA, underlining that relatively low-dose auxin and reduced salts can be optimal for bulbous plant rooting. Our rooted plantlets showed no signs of abnormal callus at the base, an important factor since excess callus can hinder nutrient uptake and transplant success [14].

We acclimatized the regenerated *M. latifolium* bulblets by gradually transferring them to a peat and peat-soil mixture. Thanks to their well-developed root systems, the transplant shock was minimized. Literature benchmarks indicate that survival rates of micropropagated *Muscari* have historically ranged from poor to moderate. For example, bulblets of *M. azureum* produced *in vitro* showed only ~14% survival after transfer to soil [8]. Even with careful techniques, Vaziri et al. [12] noted only 17% of *M. aucheri* bulblets survived acclimatization. An important breakthrough came from Faruq et al. [14], who reported about 60% survival for *M. armeniacum* plantlets by using bulblets that had no basal callus and well-formed roots [14]. In our study, we achieved survival frequencies that approach those higher figures, attesting to the improved hardiness of our plantlets. 47% of our *in vitro*-raised *M. latifolium* plants established successfully in peat-soil mixture (under greenhouse conditions and later outdoors), a rate closer to Faruq's [14] results and markedly higher than earlier *Muscari* reports. This success can be attributed to the combination of our protocol's elements -starting with genetically intact seeds, avoiding prolonged callus phases that might weaken regenerants, ensuring strong root development, and employing a staged acclimatization process.

4 CONCLUSION AND SUGGESTIONS

A micropropagation protocol was established using stems derived from axenic bulblets of *M. latifolium* that were germinated on MS medium. It represents the first detailed report on regeneration from seed-derived explants for this species. For *M. latifolium* seeds, it is recommended to prefer 20% NaOCl concentration to provide the optimum germination and sterilization balance. 30-minute application of 20% NaOCl can be recommended as the most effective surface sterilization protocol as it completely prevents infection and provides 100% germination rate. The combination of 1.0 mg/l BAP produced the highest number of bulblets. the highest rooting percentage (100%) was achieved on MS medium supplemented with 2.0 mg/l IBA. Acclimatization trials demonstrated that transferring plantlets to a peat-soil mixture resulted in a 47% survival rate under greenhouse conditions. Our findings demonstrate that seed-derived bulblet formation is a viable alternative to traditional bulblet-based *in vitro* culture methods. This study highlights that a seed-based approach can successfully generate bulblets, making it particularly valuable for conservation efforts where minimizing wild collection is a priority and establishes a reliable *in vitro* propagation protocol for *M. latifolium*, providing a foundation for its ex situ conservation and sustainable population restoration. The findings will facilitate the enhanced utilization of the species as an ornamental plant through simplified propagation methods, while also contributing to its conservation by minimizing its extraction from natural habitats.

Acknowledgements

I like to thank Prof. Dr. Engin TİLKAT (Batman University) and Assistant Prof. Dr. Veysel SÜZERER (Bingöl University) for their helpful suggestions, encouraging conversations, and constructive support whenever I needed it during the project. This study is a part of the doctoral thesis of Nesrin BOZHAN (Dicle University).

Conflict of Interest Statement

There is no conflict of interest between the authors.

Statement of Research and Publication Ethics

The study is complied with research and publication ethics.

Artificial Intelligence (AI) Contribution Statement

Artificial intelligence (AI) tools were used solely for language editing, grammar corrections, and improving the fluency of the manuscript. All content, including text, data analysis, and figures, was solely generated by the authors.

Contributions of the Authors

Nesrin BOZHAN: Conceptualization, Data Curation, Formal analysis, Investigation, Methodology, Resources, Visualization, Writing – original draft, Writing – review and editing.

Ahmet ONAY: Conceptualization, Methodology, Project administration, Writing – review and editing.

REFERENCES

[1] H. Yılmaz, O. Y. Yılmaz and Y. F. Akyüz, "Determining the factors affecting the distribution of *Muscari latifolium*, an endemic plant of Turkey, and a mapping species distribution model," *Ecology and Evolution*, vol. 7(4), pp. 1112-1124, 2017.

[2] C. A. Ozel, K. M. Khawar and F. Unal, "Factors affecting efficient in vitro micropropagation of *Muscari muscarimi* Medikus using twin bulb scale explants," *Saudi Journal of Biological Sciences*, vol. 22(2), pp. 132-138. 2015.

[3] J. Ma, Z. Li, and Y. Liu, "Integrating Multi-Omics Analysis Reveals the Regulatory Mechanisms of White-Violet Mutant Flowers in Grape Hyacinth (*Muscari latifolium*)," *International Journal of Molecular Sciences*, vol. 24(5), pp. 5044, 2023.

[4] P. H. Davis, and D. C. Stuart, *Muscari* Miller. Davis P.H (Ed.), *Flora of Turkey and the East Aegean Islands*, vol. (8), pp. 245-263, Edinburgh: Edinburgh University Press. 1984.

[5] C. A. Ozel, "*In Vitro* Bulb Production in Different *Muscari* Species", *Doctoral Thesis*, Gazi University, Institute of Science, Ankara, 2008.

[6] V. Sarasan, R., Cripps, M. M. Ramsay, C. Atherton, M. McMichen, G. Prendergast, and J. K. Rowntree, "Conservation in vitro of threatened plants-Progress in the past decade" *In Vitro Cellular & Developmental Biology-Plant*, vol. 42(3), pp. 206-214, 2006.

[7] S. Jevremović, A. Subotić, M. Trifunović, and M. Nikolić, "Plant regeneration of Southern Adriatic iris by somatic embryogenesis," *Archives of Biological Sciences*, vol. 61(3), pp. 413-418. 2009.

[8] S. Uranbey, A. Ipek, M. Caliskan, E. Dundar, S. Cocu, D. Basalma, and H. Guneylioglu, "*In vitro* bulblet induction from bulb scales of endangered ornamental plant *Muscari azureum*," *Biotechnology & Biotechnological Equipment*, 24(2), 1843-1848, 2010.

[9] A. G. Nasırcılar, S. Mirici, U. O. Karaguzel, O. Eren, and I. Baktır, "*In vitro* propagation of endemic and endangered *Muscari mirum* from different explant types," *Turkish Journal of Botany*, vol. 35(1), pp. 37-43, 2011.

[10] R. Karamian, A. Sharifzadeh, and M. Ranjbar, "Evidence of somatic embryogenesis for plantlet regeneration in *Muscari neglectum* Guss.," *African Journal of Agricultural Research*, vol. 6, pp. 3247-3251, 2011.

[11] B. B. Yucesan, F. Cicek, and E. Gurel, "Somatic embryogenesis and encapsulation of immature bulblets of an ornamental species, grape hyacinth (*Muscari armeniacum Leichtlin* ex Baker)," *Turkish Journal of Agriculture and Forestry*, vol. 38(5), pp. 716-722, 2014.

[12] P. A. Vaziri, S. Uranbey, and C. Sancak, "Efficient *in vitro* microprapagation for the conservation of endemic and endangered aucher-eloy grape hyacinth [*Muscari Aucheri* (boiss.) Baker]," *Journal of Applied Biological Sciences*, vol. 8(1), pp. 80-83, 2014.

[13] F. A. Ozdemir, O. Kılıç, and E. Bagcı, "*In vitro* bulb regeneration from stem explants of endemic geophyt *Muscari aucheri* (Boiss.) Baker.," *International Journal of Secondary Metabolite*, vol. 4(3, Special Issue 1), pp. 50-54, 2017.

[14] M. O. Faruq, M. Shahinozzaman, M. A. K. Azad, and M. N. Amin, "*In vitro* propagation of a cut flower variety *Muscari armeniacum* Leichtlin ex Baker through direct bulblet proliferation pathways," *GSC Biological and Pharmaceutical Sciences*, 5(1), 67-75, 2018.

[15] G. Ozturk, "*In Vitro* Propagation of *Muscari* (*Muscari neglectum*) Bulbs," *MAS Journal of Applied Sciences*, vol. (7), pp. 1160-1170, 2022.

[16] T. Murashige, and F. Skoog, "A revised medium for rapid growth and bioassays with tobacco tissue cultures," *Plant Physiol*. Vol. 15, pp. 473-497, 1962.

[17] A. A. Abdul-Baki, and G. M. Moore, "Seed disinfection with hypochlorites: A selected literature review of hypochlorite chemistry and definition of terms," *Journal of Seed Technology*, pp. 43-56, 1979.

[18] E. F. George, M. A. Hall, and G.J. De Klerk, "Plant propagation by tissue culture," 3rd Edition. The Netherland, *The Back Ground Springer*, 65-175, 2008.

[19] C. Telci, M. Yildiz, S. Pelit, B. Onol, E.G. Erkilic, and H. Kendir, "The effect of surface-disinfection process on dormancy-breaking, seed germination, and seedling growth of *Lathyrus chrysanthus* Boiss. under *in vitro* conditions," *Propagation of Ornamental Plants*, vol. 11(1), pp. 10-16, 2011.

[20] M. Hesami, M. H. Daneshvar, and A. Lotfi-Jalalabadi, "Effect of sodium hypochlorite on control of *in vitro* contamination and seed germination of *Ficus religiosa*," *Iranian Journal of Plant Physiology*, vol. 7(4), pp. 2157-2162, 2017.

[21] M. Yildiz, and H. Ekiz, "The effect of sodium hypochlorite solutions on *in vitro* seedling growth and regeneration capacity of sainfoin (*Onobrychis viciifolia* Scop.) hypocotyl explants," *Canadian Journal of Plant Science*, vol. 94(7), pp. 1161-1164, 2014.

[22] B. E Lindsey III, L.Rivero, C. S. Calhoun, E. Grotewold, E. And J. Brkljacic, "Standardized method for high-throughput sterilization of *Arabidopsis* seeds," *Journal of visualized experiments: JoVE*, 128: 56587, 2017.

[23] A.I. Hsiao, "The effect of sodium hypochlorite, gibberellic acid, and light on seed dormancy and germination of wild buckwheat (*Polygonum convolvulus*) and cow cockle (*Saponaria vaccaria*)," *Canadian Journal of Botany*, vol. 57(16), pp. 1735-1739, 1979.

[24] A. Tunç, A. ve S.S. Yılmaz, "Effect of Different Explant Sources, Metatopoline and PEG on *In Vitro* Propagation of *Pistacia terebinthus* L.," *Journal of Kırşehir Ahi Evran University Faculty of Agriculture*, vol. 3(2), pp. 200-212, 2023.

[25] M. A. K. Azad, and M. N. Amin, "Effects of hormonal and basal nutrient medium on *in vitro* regeneration of an ornamental plant-*Muscari armeniacum Leichtlin*. ex Baker.," *Plant Tissue Culture and Biotechnology*, vol. 22(2), pp. 113-126, 2012.

[26] R. B. Malabadi, and J. Van Staden, "Regeneration of *Ornithogalum in vitro*," *South African Journal of Botany*, vol. 70, pp. 618-621, 2004.

[27] J. Suh, W. Lee,and A. Lee, "New plantlet proliferation and bulbing promotion in vitro cultures of *Ornithogalum* hybrid," *Acta Horticulturae*, vol. 683, pp. 155-16, 2005.

[28] S. Uzun, I. Parmaksız, S. Uranbey, S. Mirici, E. O. Sarıhan, A. Ipek, M. D. Kaya, B. Gurbuz, N. Arslan, C. Sancak, K. M. Khawar, and S. Ozcan, "*In vitro* micropropagation from immature embryos of the endemic and endangered *Muscari muscarimi* Medik.," *Turkish Journal of Biology*, 38(1), 83-88, 2014.

[29] A. Fida, "*In Vitro* Propagation of *Muscari Neglectum* Guss. Commonly Found in Van Region," *Master's Thesis*, Van Yüzüncü Yıl University, Institute of Science, Van, 2020.