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Research Article

Induction of Adventitious Bulb Regeneration via Leaf Explants of *Muscari neglectum*

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Abstract

In this study, shoot organogenesis and micropropagation were derived from *in vitro* from leaf explants of *Muscari neglectum*. Leaf explants were cultured in Murashige and Skoog (MS) medium containing TDZ (Thidiazuron) and BA (Benzyladenine) at concentrations of 0.1-0.5 mg L^{-1} combination with 0.25 mg L^{-1} NAA (α-naphthalene acetic acid) in cytokinin medium for adventitious bulblet regeneration. The highest means were obtained in 0.5 mg L^{-1} TDZ + 0.25 mg L^{-1} NAA in terms of shoot number (23.0), shoot length (1.9), root number (1.0) and root length (1.1 cm). The shoots developed from leaf explants were sub cultured in rooting. The media containing 1.0 mg L^{-1} indole-3-butyric acid (IBA) had the highest mean for plantlets number (3.0), plantlets length (6.3 cm), root number (5.0) and root length (2.0 cm). Then regenerated plantlets from *in vitro* were acclimatized under *ex vitro* in the greenhouse.

Keywords: TDZ, NAA, IBA, regeneration, micropropagation

1. Introduction

Flower bulbs, which have fleshy scales that store nutrients, constitute biennial or perennial herbaceous plants that have changed into bulbs, tubers and rhizomes to store nutrients, which we call underground organs. *Muscari,* which is among the geophytes, attracts attention with its beautiful blue-purple flowers. It is also called 'Arabian hyacinth' and 'mountain hyacinth' among the public (Uysal, 2002). Muscari species are generally perennial, herbaceous form and bulbous plants that bloom in spring, with the gene center being Anatolia (Davis and Stuart, 1984; Dahlgren et al., 1985; Dalgic, 1990; Dane, 2006; Ilcim et al., 2020). This bulbous plant species is ecologically adapted to forest ecosystems. It grows naturally in forest understories and open areas, contributing to the conservation of biological diversity in these habitats. It is not selective in its cultivation area and can grow naturally in very wide areas, from tree shadows to stony areas. *Muscari Miller* species include plants with medicinal uses as well as ornamental, aromatic and vegetable plants (Tanker et al., 1998). Medicinal uses are mostly recorded for the species *M. comosum*. *M. neglectum* and *M. armeniacum* are widely cultivated as garden plants. There is a loss of genetic resources due to the unconscious removal of these species in nature (Gursoy, 2016; Topal et al., 2022). However, although our country is the gene center of these species, their commercial production is not sufficient. Although they have the potential to be used as both ornamental and medicinal plants, the production of these species is not available. The reason for this is that it takes a long time, approximately 3-5 years, for *Muscari* species to propagate traditionally from seeds and bulbs (Arslan and Sarihan, 2002; Kalyoncu, 2007). However, rapid production of these species can be achieved by tissue culture (Tipirdamaz et al., 1999; Ozturk, 2021a). Thus, it may be possible to reproduce these species at the desired speed and time under controlled conditions (Hazarika, 2006; Seyidoglu, 2009). In *in vitro* cultures of these species, different explant sources (Uranbey et al., 2006; Ozturk, 2021b) are cultured, and *in vitro* stocks can also be maintained for many years (Debergh and Zimmerman, 1993; Suzuki and Nakata, 2001; Ozel et al., 2007; Ozturk, 2021c). This may enable the economic evaluation and commercial production of these species (Ozturk, 2022). *In vitro* propagation has the potential for commercial propagation of these species. *M. neglectum* is consumed as a spice or fresh for its blue flowers and bulbs and has medicinal properties (Lim, 2014). In addition, numerous studies have highlighted the antimicrobial effects and therapeutic potential of *M. neglectum* (Ozkan et al., 2017). This has led to a growing interest in faster, more practical propagation methods for both cultivation and conservation. Previous studies have explored the potential of *in vitro* culture techniques for the propagation of *M. neglectum* (Karamian et al., 2011; He et al., 2016; Ozel and Unal, 2016a, b). Tissue culture-based approaches present a promising solution for the mass multiplication of this species under controlled conditions. Such techniques not only accelerate propagation but also facilitate the preservation of genetic resources. Nonetheless, there remains a pressing need to establish more effective regeneration systems through optimized protocols tailored to *M. neglectum*, in order to enhance its availability for both *in vitro* and commercial applications. There has been no report on *in vitro* propagation of *M. neglectum* using leaf explants (Ozturk, 2022). Therefore, this study aims to establish a reproducible micropropagation strategy by utilizing leaf explants cultured on Murashige and Skoog (MS) medium supplemented with two cytokinin of Thidiazuron (TDZ) and Benzyl Adenine (BA) in combination with α-naphthalene acetic acid (NAA). It was then intended to root the cuttings obtained in a medium containing different doses of IBA.

2. Material and Methods

Plant material and disinfection *M. neglectum* leaf explants were used as genetic material in the study. Leaf explants were cultured in Ege University Faculty of Agriculture Department of Field Crops Tissue Culture Laboratory to determine their

regeneration abilities in different nutrient media under *in vitro* conditions. Leaf explants of *M. neglectum.* collected from a forest were kept in bleach for 3 hours in the laboratory before culture, and then they were kept in distilled water. For surface disinfection of *M. neglectum* leaf explants, they were kept in 2.5% NaClO (Sodium hypochlorite), 70% ethyl alcohol and distilled water for 4 minutes.

2.1. Adventitious bulblet proliferation

Leaf explants of approximately 0.3-0.5 cm in size were cultured *in vitro*. MS medium without plant growth regulators was used as a control. MS medium supplemented with 0.1 and 0.5 mg L^{-1} Thidiazuron (TDZ) / Benzyl Adenine (BA) was prepared together with 0.25 mg L^{-1} α Naphthalene acetic acid (NAA) for adventitious shoot induction. The medium was prepared with 2% (w v^{-1}) sucrose and 0.6% (w v^{-1}) agar (Sigma). The pH of the medium was adjusted 5.7 ± 0.1 before autoclaving at 118 kPa. Culture room conditions were at 23±2 ºC and 1500-2000 lux provided by under a 16 h light and 8 h dark cycle with cool fluorescent tubes (1.35 µMol (photons) $s^{-1}m^{-2}$). *M. neglectum* leaf explants were cultured for *in vitro* shoot development. A total of 60 leaf explants, 5 for each medium were cultured according to the amount of material collected from nature, using a completely randomized plot design, with three tubes and two replicates for each medium. The regeneration of *M. neglectum* leaf explants was performed successfully after three weeks, and the parameters of shoot number, shoot length, root number, and root length were evaluated.

2.2. *In vitro* rooting

Shoots 2-3 cm long developed from *in vitro* leaf explants were cultured in a nutrient medium prepared at concentrations of 0.1, 0.5, 1.0, and 2.0 mg L^{-1} of MS+ IBA (Indole-3-Butyric acid) for root formation. After three weeks, plantlets were transferred to fresh medium of the root composition and cool white fluorescent light: 100 Lux x 0.0135=1.35 µMol (photons) $s^{-1}m^{-2}$ for. 16 h photoperiod at 24 °C. The culture process was carried out in the Completely Randomized Plot Design with 3 replications. 10 explants were cultured for each medium. The root of *M. neglectum* shoot explants was performed successfully after three weeks, and the parameters of plantlet number, plantlet length, root number, and root length were evaluated. Stages of *in vitro* regenera *M. neglectum* leaf explants in BA and TDZ medium were given in Figure 1.

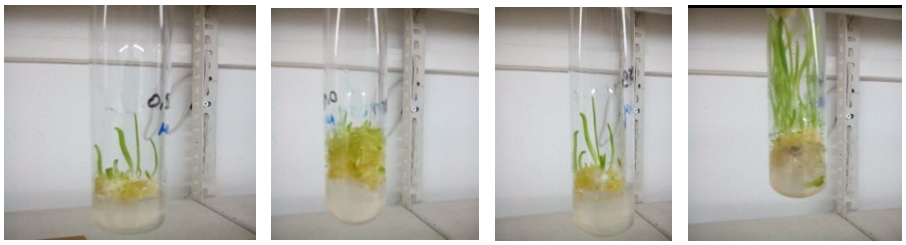


Figure 1. Stages of *in vitro* regeneration *M. neglectum* leaf explants in BA and TDZ medium

2.3. Data analysis

All data obtained for organogensis media and rooting media from leaf explants were analyzed by the standard technique of Analyses of Variance (ANOVA) and the means for shoot number, shoot length (cm), number of roots and root length were compared by using the LSD test by Steel and Torrie (1980).

3. Results and Discussion

3.1. *In vitro* direct adventitious bulblet proliferation from the leaf explants

There were statistical differences at the $p \leq 0.01$ level between the control and 4 different nutrient media in terms of the number of shoots, shoot length (cm), number of roots and root length (cm). Direct adventitious shoot induction occurred to different levels in the media (Table 1). When the nutrient media in

Table 1 were evaluated in terms of the number of shoots, the highest number of shoots was obtained with 23.0 in the medium containing 0.5 mg L^{-1} TDZ. In terms of shoot length, the two media containing 0.5 mg L^{-1} BA (2.2 cm) and TDZ (1.9 cm) were the highest. For root number and root length, the medium containing 0.5 mg L^{-1} TDZ was found to be high, with 1.0 and 1.1 cm, respectively. However, there was no growth in without PGRs and media containing 0.1 mg L^{-1} BA in terms of shoot regeneration (Table 1).

Table 1. Means of shoot number, shoot length (cm), number of roots and root length of *Muscari neglectum* leaves in different nutrient media

Number of medium	Cultures	Shoot number	Shoot length (cm)	Root number	Root length (cm)
PGR1	MS (Control)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
PGR2	0.1 mg L^{-1} TDZ	15.0 ± 0.58 ^{c}	1.1 ± 0.10 ^{c}	**1.0 ± 0.14** ^{a}	0.3 ± 0.04 ^{b}
PGR3	0.5 mg L^{-1} TDZ	**23.0 ± 0.86** ^{a}	**1.9 ± 0.11** ^{b}	**1.0 ± 0.06** ^{a}	**1.1 ± 0.07** ^{a}
PGR4	0.1 mg L^{-1} BA	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
PGR5	0.5 mg L^{-1} BA	8.0 ± 0.37 ^{b}	**2.2± 0.12** ^{a}	0.0 ± 0.0	0.0 ± 0.0
$LSD_{0.05}$		2.300	0.501	0.081	0.182
F		246.750**	53.329**	571.000**	90.800**

**: Means ± standard errors within a column followed by the same letter are not significantly different according LSD multiple range test at p≤0.05

Saniewski and Puchalski (1987) determined that low concentrations of Jasmonic acid delayed growth in *M. italica* species, whereas high concentrations of BAP increased bulb formation. Kromer (1989) *M. racemosum* L. Mill. in his study on the plant, he reported that leaves as explants provided adventitious bulb formation and root formation at a rate of 8% in the dark and in MS nutrient medium. Kromer and Kukulczanka (1992), *Muscari botryoides* Mill. in their study on plants, Kromer and Kukulczanka (1992) reported that the combination of 1-2 mg L^{-1} BAP and 0.2-2 mg L^{-1} IBA was the best for adventitious shoots. When comparing media containing BAP and TDZ in *in vitro* regeneration, it has been reported that more shoots were obtained in containing TDZ (Mok and Mok, 1985; Vinocur et al., 2000). Suzuki and Nakano (2001) reported a high rate of callus formation in *M. armeniacum* in medium containing 2-4 D, NAA and picloram. Mirici et al. (2005) reported that media containing high cytokinins and low auxin were suitable for *in vitro* regeneration of different geophyte species. Ozel (2008) reported that *M. neglectum*, 100% bulb formation was observed in the combination of 0.1 mg L^{-1} TDZ-2 mg L^{-1} NAA. Uranbey et al. (2009) reported that the highest bulb formation in the same species was from the medium containing 1 mg L^{-1} Kn, with 34.5 pieces. Nasircilar et al. (2011) examined the bulb formation of different explant sources of *M. mirum* and obtained 23.50 from the medium of onion leaves containing 4 mg L^{-1} BA and 0.25 mg L^{-1} (NAA). In their study, Azad and Amin (2012) cultured *M. armeniacum* in media containing BAP and Kinetin and reported that the best regeneration was in the medium containing 4.0 µM BAP. Uzun et al. (2014) reported that MS media supplemented with 4.0 mg L^{-1} BA and 0.50 mg L^{-1} NAA produced the highest bulb regeneration after 1 year in culture. Ozel et al. (2015) stated that MS medium containing BA and NAA was found to have the best performance for *in vitro* corm regeneration in *M. muscaimi* twin-scale explants. Ozel and Unal (2016b) cultured *M. neglectum* in containing TDZ+NAA and obtained the highest bulb ratio of 8.25. Faruq et al. (2018) in their study in which *M. armeniacum* leaf explant were cultured in different MS medium and media containing different PGRs, BA showed the best performance when it was combined with NAA. Ozel and Unal (2016a) cultured *M. neglectum* bulb in media containing different concentrations of BAP + NAA and reported that the best result was in media 4 mg L^{-1} BAP + 0.5 mg L^{-1} NAA. In his study with *M. neglectum* bulb explants, Ozturk (2022) obtained the highest number of bulb

with 8.2 in the medium containing 4.0 mg L^{-1} TDZ. Our study shows that the 0.5 mg L^{-1} TDZ medium has a higher regenerative capacity for leaf explants of *M. neglectum* for the direct production of adventitious bulblet proliferation. This finding is consistent with several previous reports (Ozel 2008; Ozel and Unal, 2016b; Ozturk 2022). Based on the present results, between the two cytokinins, TDZ was found to have better *in vitro* regeneration than BA. Vinocur et al. (2000) showed similar results in *M. botryoides.* In *in vitro* conditions, factors such as genotype, nutrient medium content, culture conditions and growth regulators affect the regeneration ability. In our study, combinations of MS+TDZ and MS+BA containing 0.25 mg L^{-1} NAA were used in the regeneration medium, and the medium containing 0.5 mg L^{-1} TDZ showed high regeneration with 23.0. In our study, leaf explants of approximately 0.3-0.5 cm in size were used and positive results were obtained in terms of shoot formation. The regeneration pathway is strongly dependent on the genotype and the concentration of endogenous PGRs in the explants. The highest cytokinin concentrations negatively affected the formation of some *Muscari* species. In these studies, TDZ was found to be an effective PGR for the induction of shoots. In addition, the risk of contamination in culture with leaf explants was less than that with bulb explants and was found suitable for *in vitro* propagation. In this study, in which the potential of obtaining a complete plant from *M. neglectum* leaf explants was investigated, media containing MS+ 0.5 mg L^{-1} TDZ/BA combined with 0.25 mg L^{-1} NAA were found to be successful in terms of direct adventitious bulblet. Regeneration could not be achieved in control and media containing 0.1 mg L^{-1} BA.

3.2. *In vitro* rooting from proliferated bullblets

There were statistical differences at the $p \leq 0.01$ significance level in terms of plant length (cm), and root length characteristics, and at the $p \leq 0.05$ significance level in terms of the number of plants and root length. Root induction occurred to different degrees in all media (Table 2). Media containing MS+1.0 mg L^{-1} IBA and 2.0 mg l^{-1} IBA were found to be the best in terms of plantlets number and plantlets length. In terms of root number, the medium containing 1.0 mg L^{-1} IBA was found to be the highest with 5.0. For root length, media containing 0.1 and 1.0 mg L^{-1} IBA were found to be high at 2.0 cm. The rooting efficiency of *M. neglectum* plantlets, expressed as the mean of root number per plantlet, varied significantly among the IBA treatments. These results clearly demonstrate that exogenous IBA is essential for increasing root productivity in *M. neglectum*, and that the application of 0.5 to 1.0 mg L^{-1} IBA provides the most favorable outcomes for *in vitro* rooting. In root cultures of different *Muscari* species, Faruq et al. (2018) in medium 2.0 µM IBA; Azad and Amin (2012) in media containing 5.0-4.0 µM IBA, NAA and IAA; Ozel and Unal (2016a) in media containing 2.0 mg L^{-1} BAP-2 mg L^{-1} NAA; Ozturk (2022) reported that the best root formation was achieved in media 2 mg L^{-1} IBA. The results obtained in our study are similar to Faruq et al. (2018) and Ozturk (2022), in line with Azad and Amin (2012); It was found to be partially compatible with Ozel and Unal (2016a). Based on the present results, among the different auxin levels, 0.1 mg L^{-1} IBA medium contributed to higher root formation for all traits. After rooting of shoots for three weeks were cultured to the rooting medium to obtain a complete plant, and the media containing MS+ 1.0 and 2.0 mg L^{-1} IBA were found to be successful in terms of root number and root length. Considering the economy and ease of operation, medium containing MS+ 1.0 mg L^{-1} IBA can be recommended for both shoot and root development for a complete plant formation under *in vitro* conditions.

Table 2. Means of plantlets number, plant length (cm), number of roots and root length in sub-culture media of shoots developed by the regeneration of *Muscari neglectum*

Number of medium	Cultures	Plantlets number	Plantlets length (cm)	Root number	Root length (cm)
R1	MS (Control)	1.0 ± 0.26 ^{c}	1.2 ±0.06 ^{b}	1.0 ± 0.03 ^{c}	0.9 ± 0.03 ^{b}
R2	0.1 mg L^{-1} IBA	1.7 ± 0.08 ^{bc}	2.0 ± 0.08 ^{b}	2.0 ± 0.07 ^{c}	**2.1 ± 0.08** ^{a}
R3	0.5 mg L^{-1} IBA	2.3 ± 0.13 ^{ab}	2.4 ± 0.07 ^{b}	3.7 ± 0.16 ^{b}	1.1 ± 0.06 ^{b}
R4	1.0 mg L^{-1} IBA	**3.0 ± 0.04** ^{a}	**6.3 ± 0.09** ^{a}	**5.0 ± 0.09** ^{a}	**2.1 ± 0.10** ^{a}
R5	2.0 mg L^{-1} IBA	**3.0 ± 0.13** ^{a}	**4.9 ±0.09** ^{a}	4.7 ± 0.07 ^{ab}	1.5 ± 0.06 ^{ab}
$LSD_{0.05}$		1.329	1.994	1.329	0.877
F		4.250*	11.410**	16.688**	3.656*

**: Means ± standard errors within a column followed by the same letter are not significantly different according LSD multiple range test at $p \leq 0.05$.

*: Means ± standard errors within a column followed by the same letter are not significantly different according LSD multiple range test at $p \leq 0.05$.

4. Conclusions

The potential of leaf explants of *Muscari neglectum* for adventitious bulblet regeneration was investigated in this study. The findings demonstrated that different combinations of cytokinins and auxins played a crucial role in the regeneration process. TDZ exhibited a higher regenerative capacity compared to BA, confirming its suitability for leaf explants. During the rooting phase, IBA proved to be the most effective auxin. The results indicate that this concentration supports balanced shoot and root development and represents the most suitable medium for obtaining complete plants under *in vitro* conditions. Overall, the findings represent a significant contribution to the advancement of *in vitro* propagation protocols, the conservation of *M. neglectum*, and its potential integration into commercial production systems. Likewise, leaf explants *of M. neglectum* are suitable for *in vitro* organogenesis and microclonal production and are of great importance in the commercial production of this species.

Declaration of Author Contributions

The authors declare that they have contributed equally to the article. All authors declare that they have seen/read and approved the final version of the article ready for publication.

Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

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