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Original scientific article

EVIDENCE OF EFFICIENT *in vitro* MICROPROPAGATION FROM BULB SCALES OF STARCH GRAPE HYACINTH (*Muscari neglectum* Guss. Ex. Ten.)

Abdullah FİDA[1], Nurhan KESKİN[2*], Parisa Pourali KAHRİZ[3], Mahsa Pourali KAHRİZ[3], Khalid Mahmood KHAWAR[4]

[1]Department of Horticulture, Graduate School of Natural and Applied Sciences,Van Yüzüncü Yıl University Van, Türkiye
Department of Horticulture, Faculty of Agriculture, Van Yüzüncü Yıl University, 65080, Van, Türkiye
[3]Department of Field Crops, Graduate School of Natural and Applied Sciences, Ankara University, Ankara, Türkiye
[4]Department of Agriculture and Food Hemp Research Institute, Yozgat Bozok University, Yozgat, Türkiye

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Muscari neglectum is a widespread plant species that grows in various areas of the Mediterranean region in the wild. The species has a high potential for use as cut flowers, outdoor or indoor ornamental or garden plants. It is also used as a food, and forage plant on limited scales in rural areas. There is a lack of commercial production protocols for *M. neglectum*. The development of a fast and efficient protocol for commercial production, may contribute to the economy of Türkiye and improve local people's quality of life. Therefore, this study aimed to develop safe *in vitro* vegetative propagation techniques using 2, 3, 4, and 5 bulb scale explants on MS medium using 1 mg BAP L^{-1} + 0.00, 0.40, 0.60, 0.80, 1.00,1.20 mg NAA L^{-1}. Any number of bulb scale explants obtained from freshly harvested bulbs were not suitable for micropropagation and developed necrosis. However, any number of bulb scales obtained from 6 weeks of waiting for bulbs did not show the problem. A regeneration percentage of 13.33 to 100% was noted on 2-scale explants. Additionally, 100% callus formation was observed on these scales using MS

Corresponding author: Nurhan Keskin, Department of Horticulture, Faculty of Agriculture, Van Yüzüncü Yıl University, 65080, Van, Türkiye, E-mail: keskin@yyu.edu.tr
Abdullah Fida ORCID:0000-0001-5254-8260;Nurhan Keskin ORCID:0000-0003-2332-1459;Parisa Pourali Kahriz ORCID:0000-0002-6420-7225;Mahsa Pourali Kahriz ORCID:0000-0002-2471-3799;Khalid Mahmood Khawar ORCID:0000-0001-5110-6014

medium containing 1 mg BAP L^{-1} + 0.8, 1.0, and 1.20 mg NAA L^{-1} (3 combinations). In addition, 1.93 bulblets with a diameter of 0.97 cm (the largest) were observed on MS medium containing 1 mg BAP L^{-1} + 0.4 mg NAA L^{-1}. Although, 3, 4, and 5 bulb scale explants induced bulblets in variable numbers, the rate of bulblet induction was not at par with bulblet induction on 2 scale explants. The largest bulblets developed on 2-scale explants were rooted on ½ × MS medium containing 0.5 mg NAA L^{-1}. The results obtained for micropropagation of *M. neglectum* species are of great importance for the commercial production of the species.

Keywords: Bulb scales, bulblets, ornamental plants, rooting

INTRODUCTION

Muscari Mill. (Asparagaceae) is a genus of ornamental bulbous geophytes with wide distribution in the Mediterranean region including Central, Eastern, and Southeastern Anatolia (DEMIRCI *et al*., 2013, KAYIRAN and ÖZHATAY 2017). They also grow in some parts of Europe, Iraq, Caucasia, Iran, Central Russia, Pakistan, and Afghanistan (SPETA, 1998; JAFARİ *et al*., 2008, FOP, 2020).

Fifty (50) Muscari species grow in the world (SPETA, 1998) and out of 37 Muscari species growing in Türkiye 25 species are endemic (KAYIRAN and ÖZHATAY, 2017). All Muscari species including, *Muscari neglectum* Guss. ex Ten. (starch grape hyacinth) are herbaceous meaty bulbous geophyte species that take length of approximately 4-30 cm and grow under high temperatures up to 35 °C or more in shade or open and flower from March to May every year (SEÇMEN et al., 1995; TUBİVES, 2020). It could survive with extremely poor and limiting groundwater conditions in dry environments on semi-arid or arid patches of marginal poor-quality soils made up of sand, calcareous rocky slopes, dunes, or (rarely) meadows at an altitude of 0-2300 m above sea level (DAVIS and STUART, 1984; ÖZHATAY *et al*., 2009, 2011, 2013, 2015; DEMİRCİ *et al*., 2013; TUBİVES, 2020). This plant species also prevents degradation and soil erosion by holding it as a stabilizer.

They could be widely used as cut flowers, or for indoor and outdoor ornamental purposes. The use of hard drought drought-resistant cheap local ornamental plants is desired in urban landscaping (IŞIK, 2005). Therefore, they can be used for the creation of visual and functional effects primarily on urban ecology by filling the open and green spaces between the petrified and concrete masses of the cities (BOOTH *et al*., 2004; BOOTH, 2005). This way they can also contribute to increasing and improving life standards ensuring environmental safety in a cheap way.

The extraction of geophytes from the natural environment and their export have been prohibited or restricted to quota to avoid their destruction and conservation through laws promulgated by the Republic of Türkiye Ministry of Agriculture and Forestry, as published in the OFFİCİAL GAZZETE (2017) and earlier. However, due to difficulties in the implementation of laws in far-off regions; these plant species are being removed haphazardly by enthusiasts for selling in local floral markets which represents a significant threat to their existence.

Although, *M. neglectum* grows in a large stretch of land, in Türkiye through seeds or through 1-2 mm diameter 6-10 propagules (depending on the size of the mother bulb) on the periphery of the basal plate. It grows as a wild plant besetting their slow and poor regeneration

levels in nature. Ideally, these bulblets feed on dying mother bulb/s. Thereafter, these bulblets grow up individually to take the size and are capable of blooming after 3-5 years; depending on the health status of the plants, plant nutrients of the soil, and the surrounding environmental conditions and achieve size to bloom after a long time (after 4-5 years). There is no reported technique for agronomic or *in vitro* propagation of the plants belonging to this species. Therefore, its potential for use as an ornamental plant for commercial cultivation is lacking and the plants are badly underutilized.

It is desirable to shorten both long vegetative or generative multiplication periods of bulbous plants including *M. neglectum* by introducing reliable and efficient micropropagation techniques in agreement with JEVREMOVIC *et al*. (2009) that could offer easy alternatives for commercial production and conservation of the threatened bulbous plant species.

Bulblets regeneration from about 14 Muscari species has been reported using vegetative to reproductive organs (OZEL *et al*., 2009; NASİRCILAR *et al*., 2010; UZUN *et al*., 2014). Most of the successful regeneration reports of Muscari species have focussed on *M. armeniacum. M. neglectum* are threatened and vulnerable requiring protection. A review of literature shows only two reports of bulblet regeneration using somatic embryogenesis from bulbs and protoplast cultures of *M. neglectum* (KARAMIAN *et al*., 2011a; KARAMİAN and RANJBAR, 2011b).

Türkiye has an important place in the export of flower bulbs. The development of a systematic multiplication procedure could be helpful in both protecting and contributing to these plants to the national and local economies of the farmers.

Techniques that ensure the proliferation of fast, effective, and rapid production under *in vitro* conditions are very important and desired. Therefore, a systematic approach to exploit alternative *in vitro* micropropagation techniques for their large-scale production would be useful.

The objective of this study was to identify and optimize possibilities of *M. neglectum* 2, 3, 4, and 5 bulb scales for bulblet regeneration under *in vitro* conditions.

MATERİALS AND METHODS

Plant material

M. neglectum bulbs were picked and collected from the campus of the Van Yüzüncü Yıl University, Türkiye; where they grow profusely under wild conditions. These were jointly identified by Associate Professor Dr. Nurhan Keskin of the Department of Horticulture of the same University and Dr. Parisa Pourali Kahriz of Ardahan University, Türkiye very carefully. The plants were identified using identification keys given in Flora of Pakistan (FOP, 2020), Flora of North America (FONA, 2020), and the Ornamental Plants From Russia And Adjacent States of The Former Soviet Union (OPRASFSU, 2020).

Surface sterilization of bulbs

First of all disease-free, unbruised, and undamaged bulbs of *M. neglectum* were sorted. These bulbs were washed with commercial detergent and dried. They were divided into two sets. The first set was kept in a dry, dark, and cool storage place (20°C) for six weeks. The second set was subjected to surface sterilization using a few drops of Tween 20 added to 4% commercial bleach (5% NaOCl) for 20 min using a magnetic stirrer. Thereafter, these were carefully rinsed

for 3 × 5 min with autoclaved and sterilized distilled water. The first set of bulbs was also subjected to 6 weeks of rest in a dark and cool place.

Explants

The explants from fresh and six weeks rested bulbs were obtained similarly as described in the following lines. After, removing the outer flakes and roots of the sterilized bulbs, the bulbs were vertically sliced into four pieces to obtain 2, 3, 4, and 5 scales from the slices such that the lower portion of each explant was attached to a minor attachment of the basal plate.

These were cultured on 7 g L^{-1} agar (Sigma Type A) solidified MS medium fortified with 60 g L^{-1} sucrose in sterile Petri dishes for 4 days and incubated under 16 hours light and 8 hours dark photoperiod at 24 ± 1°C as a control to check the incidence of fungal or bacterial contamination.

These were carefully selected and uncontaminated explants were cultured on MS medium using 1 mg BAP L^{-1} with 0, 0.4, 0.6, 0.8, 1.0, and 1.20 mg NAA L^{-1} (6 treatment combinations). MS medium without any plant growth regulator served as a control treatment.

All bulblets were rooted on ½ × MS medium containing 0.5 mg NAA L^{-1} . All culture treatments were prepared after adjusting pH to 5.7 ± 0.1 using 0.1 N NaOH or 0.1 N HCl before subjecting them to an autoclave (Hirayama HG-50 CE Otoklav Japan) under 1.45 kPa atmospheric pressure using the temperature of 121°C for 21 min. The regenerated bulblets were transferred to 35 ml MS medium. These were exposed to 16/8 h light photoperiods at 25°C with a photosynthetic photon flux of 80 µmol photons $m^{-2}s^{-1}$ for 12 weeks until the development of leaves. These were deflated, and transferred to 2.5l transparent plastic pots containing 2.3l peat moss. The plastic pots were made by vertical slicing of a 5l bottle. These pots were placed in the greenhouse under mist; maintaining a relative humidity of ~80% and photosynthetic light intensity of 40 µmol photons $m^{-2}s^{-1}$. The humidity was gradually reduced to ~40% at 24°C after 5 weeks. The data was collected on the percentage of surviving plants to conclude the experiment.

Statistical analysis

Sixty explants were used for each treatment, these were divided into 12 equal replications; each replication contained 5 explants. Statistical analysis was done using one-way anlysis of variance using explants as the main factor and the plant growth regulator treatments as the subfactor. The means of all treatments for each parameter were compared. The mean values of the parameters shown in percentage were arc sine transformed before subjecting the data to the analysis of variance as shown by GOMEZ and GOMEZ (1984).

RESULTS

Necrosis on two bulb scales taken from fresh bulbs

Different types of bulb scale sets from fresh bulbs began to induce gradual necrosis due to exudation from micro and macroscopic injuries made to bulb tissues to obtain bulb scales after 3-4 d on all BAP + NAA cultures treatments. This appeared by showing stress symptoms,

profuse damage, and necrosis on bulb scale cells and tissues. Necrosis spread was noted on 70-80% of each type of bulb scale in 8-10 d. Consequently, no regeneration was visualized.

Regeneration on two bulb scales taken from cold stored bulbs

Callus formation percentage (%)

There was no callus formation on the MS medium (treated as control treatment) and 1 mg BAP L^{-1} with 0.00, 0.40, and 0.60 mg NAA L^{-1} (3 combinations). However, 1 mg BAP L^{-1} with 0.80, 1.00, and 1 mg NAA L^{-1} induced 100% callus formation on the MS medium 3 combinations (Table 1).

Table 1. Effects of fixed concentration of BAP and variable concentrations of NAA on bulblet regeneration of Muscari neglectum using 2 scale explant

BAP (mg L^{-1})	NAA (mg L^{-1})	Cllus induction percentage (%)**	Bulblet induction percentage (%)**	Number of shoots per explant**	Bulblet diameters (cm)*	Number of leaves per plant*	Leaf length (cm)*
1	0.00	0.00c	100.00 a	1.40 d	0.60 bc	00.00	00.00
1	0.40	0.00c	100.00 a	1.93 a	0.97 a	00.00	00.00
1	0.60	0.00c	100.00 a	2.59 b	0.70 b	0.20	3.20
1	0.80	100.00 a	75.00 b	3.36 b	0.50 d	00.00	00.00
1	1.00	100.00 a	53.33 c	4.33 c	0.14 e	00.00	00.00
1	1.20	100.00 a	40.00 d	2.55 c	0.12 e	00.00	00.00
Control (MS medium)		00.00 b	13.33d	0.33 e	0.64 bc	0.00	0.00

All values shown by different small letters in a column are significantly different using Duncan Test at $p<0.05$ level of significance

Each value is the mean of 60 explants

Bulblet regeneration percentage (%)

A significantly different rate of bulblet regeneration was observed on the 2-bulb scale explants under the influence of different plant growth regulator concentrations (Table 1). Furthermore, 100% bulblet regeneration was observed in the medium containing 1 mg BAP L^{-1} + 0.00, 0.40, and 0.60 mg NAA L^{-1} . Whereas, 1 L^{-1} BAP mg L^{-1} + 0.80 NAA mg L^{-1} , 1 mg NAA L^{-1} , and 1.20 mg NAA L^{-1} induced 75.00%, 53.33%, and 40.00% bulblets regeneration respectively. Only 13.33% regeneration of bulblets was noted on the control treatment (using MS medium).

Number of bulblets per explant

A significantly different number of bulblets per explant was observed on the two bulb scales under the influence of different concentrations of BAP + NAA (Table 1). The minimum number (0.33) of bulblets were induced on the explants using a control medium treatment. However, other treatments having MS medium fortified with 1 mg BAP L^{-1} and 0.00, 0.40, 0.60,

0.80, and 1.00 mg NAA L^{-1} had a uniform and linear increase of 0.33 to 4.33 bulblets per explant. A reduction in bulblet formation was very obvious using 1 mg BAP L^{-1} + 1 mg BAP L^{-1} or 1.20 mg NAA L^{-1} .

Diameter of induced bulblets per explant

The bulblets induced on different treatments showed significantly different bulblet diameters. The bullet diameter ranged from 0.12 to 0.97 cm (Table 1). The maximum bullet diameter was monitored on the medium containing 1 mg BAP L^{-1} and 0.4 mg NAA L^{-1}. Varying bulblet formation was observed on MS medium treatments containing 1 mg BAP L^{-1} and 0.00-1.20 mg NAA L^{-1} . The diameter of the bulbs that developed in the control medium was noted as 0.64 cm.

Number of leaves per bulblet and their length

The leaves were recorded only on the medium containing 1 mg BAP L^{-1} + 0.6 mg NAA L^{-1} , making 0.20 leaves with a mean length of 3.2 cm (Table 1).

Induced bulblets on the explants were excised and successfully cultured on MS medium containing 60 g sucrose L^{-1} that showed a conspicuous increase in their diameter to approximately 0.5-0.6 cm in 16-17 weeks.

After harvesting the bulblets once, the explants were subcultured 3-4 times. The explants showed the ability to regenerate after each without losing vitality.

Bulblet regeneration on 3, 4, and 5 bulb scale explant of M. neglectum

The largest diameter was obtained from MS medium containing 1 mg BAP L^{-1} and 0.40 mg NAA L^{-1} using twin-scale or two-scaled leaf explants.

Therefore, 1 mg BAP L^{-1} and 0.40 mg L^{-1} NAA L^{-1} containing MS medium were preferred to regenerate from 3, 4, and 5 scale explants. Variance analysis results of the data showed no variation in the regeneration behavior of explants in terms of all parameters on these 3 types of explants. Therefore, the data was not subjected to Duncan's multiple range test to separate their means and the data is given numerically in Table 2.

No callus formation was noted on any explant. All 3 types of explants induced axillary bulblet induction. The number of bulblets per explant varied from 2.89 to 3.38, with minimum and maximum diameters of 0.43 - 0.49 cm. Although shoot initials of adventitious shoots were also noted on these explants individually, their count and diameter were very few and negligible; therefore, these were not considered and mentioned in the results.

Table 2. Effects of 1 mg/L BAP and 0.4 mg NAA L^{-1} on bulblet regeneration of Muscari neglectum using 3,4,5 scale explants

Number of scales used as explant	Cllus induction percentage (%)**	Bulblet induction percentage (%)	Number of bulblets per explant	Axillary Bulblets diameter (cm)
3	00.00	100.00	2.89	0.53
4	00.00	100.00	3.00	0.59
5	00.00	100.00	3.38	0.57

Each value is the mean of 60 explants

Rooting

Rooting of *M. neglectum* bulblets was achieved on MS medium having 0.5 mg NAA L^{-1} was used for rooting adventitious bulblets and after 3 weeks, 100% 7-8 cm long roots were obtained. Acclimatization of bulblets was achieved in a potting mixture containing peat moss: clay (4:1) in the greenhouse.

DISCUSSION

Necrosis on two bulb scales taken from fresh bulbs

Micropropagation of *M. neglectum* is very important since the process can aid in the rapid production of genetically uniform plants. *M. neglectum* bulbs have a high amount of anthocyanins, flavonoids, homo-isophlavanones, spirocyclic nortriterpenoid glycosides, polyhydroxylated pyrrolizidine and alkaloids (MULHOLLAND *et al*., 2013; MAHBOUBİ and TAGHİZADEH, 2016; KAYİRAN and ÖZHATAY, 2017). Fresh tissues of bulbous plants express high phenolic exudation at the scar zone due to the activation of oxidative enzymes and oxidation of phenolic compounds at the time the bulbs are sliced, involving severing of tissue wounding (HEREDIA *et al*., 2002). This results in the accumulation of a mass of dead and collapsed cells followed by cascade chain reactions of cell damage in neighboring cells that inactivate regeneration and inhibit organogenesis in the culture medium (HEREDİA *et al*., 2001, 2002).

It is well established that all plant cells have rigid cell walls. When the fresh tissues of the explants were exposed to a hypertonic environment, containing MS medium and a number of plant growth regulators + sucrose, they ended up with plasmolysis; with complete or partial loss of physiological activities. It is likely that interaction among ROS formation from exudates of both injured and plasmolyzed the cells, increased enzyme activities, and other wear tears in tissues, which started simultaneously (KRISHNA *et al*., 2008; OZEL *et al*., 2015). and were manifested visually after 1-8 days. These results are in agreement with SAVATIN *et al*. (2014). They displayed that the appearance of injury may need a latent period before expression. This gradually led to the accumulation of lethal, and toxic compounds and encouraged changes in the body structure of the cells and tissues (KRİSHNA *et al*., 2008; UCHENDU *et al*., 2011).

Regeneration on two bulb scales taken from cold stored bulbs

Several advances and recommendations have been suggested for inhibiting or reducing oxidative damage-based necrosis in fresh cultures of plant cells and tissue cultures (KRISHNA *et al*., 2008).

Cell walls provide an excellent barrier against external threats when the cell detects any kind of potential threats. It gives structural support flexibility, and strength and is made up of cellulose, and polysaccharides using many long polymer-chained glucose monomers. They are also responsible for maintaining turgor pressure. Cold acclimation induces changes in cell wall polysaccharide composition and in the activities of cell wall-modifying enzymes (TAKAHASHİ *et al*., 2019). Cold treatments influence plant tissue metabolism, and affect a number of functions in plant cells like a decrease in their pore size (TAKAHASHI *et al*., 2019). This improves their cell wall integrity, mechanical properties, and morphology (NÄGELE *et al*., 2012; FÜRTAUER *et al*., 2019). An increase in cell wall rigidity or integrity is an important factor in tissue regeneration (LE GALL *et al*., 2015).

Some researchers have reported that necrosis could be stopped by clearing the dead cells on the tissues or by taking the explants from the mother tissue when they are in stable condition. Clearing the dead cells is not desired, as it may restart wound-induced chain of cascade reactions with increased ethylene production, ROS, and enzyme leading to the gradual accumulation of toxic compounds in the body structure of the plant tissues (KRISHNA *et al*., 2008; UCHENDU *et al*., 2011) along ROS formation leading to the death of the explant. Thus, taking explants from rested tissues enables restoration of integrity and vitality of the tissues with minimum or no damage to the explants (THORPE, 2007; HUSSAIN *et al*., 2012).

Exposure or storage of bulbs to cold storage (<10 °C temperature) for 6 weeks contributed towards these parameters (KANNERWORFF *et al*., 1994; LEE *et al*., 2002) and likely increased count of mitochondria (KHODOROVA *et al*., 2007) and energy of the tissues ending up with the ability of plant cells to overcome scar based damages amicably (AKIRA *et al*.,2006). Thus, cold treatment induced stability to the *M. neglectum* mother tissues after resting in dark for 6 weeks without allowing dysfunction and inhibition of regeneration capabilities of bulb tissues in agreement with NUNES *et al*., (2014). This stability determined the regeneration potential of the tissues after treatment with plant growth regulators. The control treatments (MS medium) with no phytohormones failed to induct new bulblets in agreement with OZEL *et al*. (2015). They had similar observations on *M. muscarimi* bulb scale explants. Considering the effect of plant growth regulator doses on bulb scale explants, the explants showed variable bulblet regeneration with and without callus induction. However, bulblet induction was not sufficient on the 3, 4, and 5 scale explants. This could be due to the bulb scale competition effect. The use of two-scale explants for the regeneration of bulblets has also been recommended by other researchers (VAZIRI *et al*., 2014; UZUN *et al*., 2014; OZEL *et al*., 2015) for the regeneration of other Muscari species.

This was a more targetted approach to reduce necrosis (OGAWA *et al*., 2016; FISCHER *et al*., 2017) before culturing them without using and involving complex techniques using antioxidants (OELZE *et al*., 2008), adsorbents (CHUTIPAIJIT and SUTJARITVORAKUL, 2018); or ethylene inhibitors (CHAE *et al*., 2012).

Acclimatisation studies

The isolated bulblets treated with sucrose were helpful to induce easy rooting and gain in diameter with and without the use of 0.5 mg NAA L^{-1} NAA in ½ × MS medium. These bulblets were very hard and were transferred to pots for acclimatization. All of them showed good acclimatization capability in agreement with (VAZIRI *et al*., 2014; UZUN *et al*., 2014; OZEL *et al*., 2015).

CONCLUSİON

This is first comprehensive report of *in vitro* bulblet regeneration from 2-5 scale bulb explants of *M. neglectum*. Based on the results of this study, it was concluded that the bulbs must be rested at low temperatures (<10°C) for some period before taking explants to thicken cell walls and minimize oxidative browning. Furthermore, 2 scale explants of *M. neglectum* could be preferably used as an efficient explant source for micropropagation. These results are of immense importance and provide a sound base that can be used for an easy commercial micropropagation approach of *M. neglectum*.

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EFIKASNA *in vitro* MIKROPROPAGACIJA IZ LUKOVICE SKROBNOG ZUMBULA (*Muscari neglectum* Guss. Ex. Ten.)

Abdullah FİDA[1], Nurhan KESKİN[2*], Parisa Pourali KAHRIZ[3], Mahsa Pourali KAHRIZ[3], Khalid Mahmood KHAVAR[4]

[1]Odsek za hortikulturu, Visoka škola prirodnih i primenjenih nauka, Univerzitet Van Iuzuncu Iıl, Van, Turska
[2]Odsek za hortikulturu, Poljoprivredni fakultet Univerziteta Van Iuzuncu Iıl, 65080, Van, Turska
[3]Odsek za ratarske kulture, Visoka škola prirodnih i primenjenih nauka, Univerzitet u Ankari, Ankara, Turska
[4]Odsek za poljoprivredu i ishranu Institut za istraživanje konoplje, Iozgat Bozok Univerzitet, Iozgat, Turska

IZVOD

Muscari neglectum je rasprostranjena biljna vrsta koja divlje raste u raznim oblastima mediteranskog regiona. Vrsta ima veliki potencijal za upotrebu kao rezano cveće ili kao spoljna ili unutrašnja ukrasna ili baštenska biljka. Takođe se koristi kao hrana i krmna biljka u ograničenim razmerama u ruralnim područjima. Nedostaju protokoli komercijalne proizvodnje za *M. neglectum*. Razvoj brzog i efikasnog protokola za komercijalnu proizvodnju može doprineti ekonomiji Turske i poboljšati kvalitet života lokalnog stanovništva. Stoga je ova studija imala za cilj da razvije bezbedne *in vitro* tehnike vegetativnog razmnožavanja korišćenjem eksplantata od 2, 3, 4 i 5 lukovica na MS medijumu koristeći 1 mg BAP L^{-1} + 0,00, 0,40, 0,60, 0,80, 1,00, 1,20 mg NAA L^{-1} . Bilo koji broj eksplantata lukovice dobijenih iz sveže ubranih lukovica nije bio pogodan za mikropropagaciju, jer se razvijala nekroza. Međutim, bilo koji broj skala za lukovice dobijen posle 6 nedelja nije pokazao problem. Procenat regeneracije od 13,33 do 100% zabeležen je na eksplantatima 2-skale. Pored toga, 100% formiranje kalusa je primećeno na ovim skalama korišćenjem MS medijuma koji sadrži 1 BAP mg L^{-1} + 0,8, 1,0 i 1,20 mg NAA L^{-1} (3 kombinacije). Pored toga, 1,93 lukovica prečnika 0,97 cm (najveće) primećeno je na MS medijumu koji sadrži 1 mg BAP L^{-1} + 0,4 mg NAA L^{-1}. Iako su eksplanti sa 3, 4 i 5 lukovica indukovali lukovice u promenljivom broju, stopa njihove indukcije nije bila ujednačena sa indukcijom na eksplantatu sa 2 skale. Najveće lukovice razvijene su na eksplantatu od 2 skale na ½ × MS medijumu koji sadrži 0,5 NAA mg L^{-1} . Rezultati dobijeni za mikropropagaciju *M. neglectum* su od velikog značaja za komercijalnu proizvodnju vrste.

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