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Efficient *in vitro* Clonal Propagation of *Muscari neglectum* Guss. Ex. Ten Using Thidiazuron- α Naphthalene Acetic Acid

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ABSTRACT

Muscari neglectum Guss. Ex Ten, is an ornamental, herbaceous perennial plant species that grows in the Mediterranean countries with attractive and scented blue-colored flowers. The plant has low seed output, seed dormancy, low germination and propagation rates. This study aimed to develop a reliable microclonal propagation protocol for *M. neglectum* using TDZ (Thidiazuron)-NAA (α Naphthalene acetic acid) to induce bulblets, roots, and acclimatization of the regenerated bulblets. Maximum number of bulblets per explant (8.25±0.05) was noted on MS medium containing 0.0454 µM TDZ-5.37 µM NAA. The bulblets regenerated in each type of culture medium were very vigorous, and acclimatized easily following rooting on a subculture. Here we show that this protocol is a useful clonal micropropagation system for this important ornamental plant.

Introduction

Muscari neglectum Guss. Ex Ten; or neglectum grape hyacinth belonging to the genus *Muscari* subgenus *Botryanthus* (Kunth) Rouy (Davis and Stuart, 1984), is an ornamental, herbaceous perennial widespread polymorphic plant species. It grows up to 2300 m altitude in the Mediterranean countries of the Eastern Europe, the North Africa and the Western Asia (Doussi and Thanos, 2002). It has lanceolate leaves about 40 cm in length and emerges in autumn, with 4-6 emerged leaves growing on the bulbs. It has attractive and scented blue-colored flowers, which are commonly used as a dressing and seasoning in Mediterranean cuisine. They are also eaten fresh or pickled, and have various nutritive and medicinal properties (Lim, 2014).

The uppermost flowers are usually light blue, small and sterile; whereas, the flowers below are blackish blue and white recurved teeth; which, bloom in spring. Its bulbs are used for its diuretic and stimulant properties. These plants are highly important medicinally, and their propagation could help in easy extraction of compounds for pharmaceutical industry (Nasrabadi et al., 2013). The root is anti-inflammatory, antiallergic and aphrodisiac with pectoral stimulating characteristics (Usher, 1974).

The plant has low seed output, seed dormancy and low germination capacity (Karamian et al., 2011). First tissue culture study on *Muscari* spp. was carried out by Saniewski and Pytlewski (1979) using leaves and inflorescence stalk of *M. comosum*. It was followed by somatic embryogenesis from leaf-derived calli by Moris and Nakano (2004). Protoplast culture-based plantlet regeneration has also been reported by Nakano et al. (2005) and Karamian et al. (2011). However, sustainable micropropagation of *M. neglectum* is elusive.

Wild flora is generally not exploited because of the lack of funds. Among these plants, many have high potential for landscaping and could be used as ornamental plants in parks and on roadsides (Jaime et al., 2016). One of these amazing plants *M. neglectum* is very difficult to propagate using conventional techniques. Moreover, their multiplication is largely affected by environmental biotic and abiotic stresses. A review of regeneration methods reported above suggest that there is a need to develop reliable microclonal propagation protocols for *M. neglectum.* The objectives of the study was to to develop a protocol for micropropagation, rooting and acclimatisation of *Muscari neglectum* using bulb twin scales as explants on MS medium containing different concentrations of TDZ (Thidiazuron)-NAA (α Naphthalene acetic acid) to establish a rapid and efficient *in vitro* propagation system. In practical terms, these microclonal propagation results will provide a useful system for a wide range of biotechnological applications for this plant species.

Materials and methods

M. neglectum bulblets were collected from Doğandede Tepesi (Beypazarı, Ankara, Turkey); where they grow in wild. The plants were identified at the department of Biology, section plant taxonomy, Gazi University, Ankara, Turkey.

Bulblets were stored in a dark, cool and dry place for 4 weeks. Thereafter, they were subjected to surface sterilisation using 80% of commercial bleach (4% NaOCl, Ace -Turkey) for 20 minutes. Each 100 ml of bleach was supplemented with 1 ml (v/v) of Tween 20 as surfactant. Sterilized experimental materials were rinsed 5 times for 3 minutes each using bidistilled sterilized water. The bulblets were sliced into four upside down vertical slices followed by separating twin bulb scales from them (slices). Each explant was attached by a thin binding segment at the basal plate. Each explant was treated with 1% (v/v) of Plant Preservation Mixture (PPM) for 2 hours. Thereafter, these bulb scales were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 7.0 g/l of Plant Cell Culture Tested type A agar (Sigma-Aldrich, Germany) and 30 g/l of sucrose (Sigma-Aldrich, Germany) for 4 days to screen them against any possible microorganism-based contaminations. All cultures were autoclaved at 121°C, 104 kPa for 20 minutes after adjusting their pH to 5.6 - 5.8.

The screened bulb scales were cultured on MS medium containing 0.0454, 0.06811, or 0.0908 µM TDZ and 2.685, 5.37, or 10.74 µM NAA.All explants were incubated at 24 ± 1°C under Philips day light lamps (TLD 36 W/54, Hungary) with a 16 h light (35 µmol photons $m^{-2}s^{-1}$) photoperiod for 8 weeks for microclonal propagation.

All rooted bulblets were washed in running tap water to remove the solidified agar medium adhering to the roots. Then, the bulblets were transferred to pots containing leaf compost and were uprooted after eight weeks. Leaf compost was made from local environment-friendly green and dried leaf material. It had low bulk density of 0.3 mg m^{-3}. Furthermore, it had a pH of 6.5 and EC of 0.3 dS m^{-1}, with porosity of ~70% v/w that allows high water absorption.

All pots containing *Muscari* bulblets were carefully covered with transparent polyethylene bags (110 guage) to maintain high relative humidity in the micro climate. Pots were kept at room temperature (24°C) conditions using Sanyo versatile plant growth chamber (). Following 12 days of acclimatization, transparent bags were perforated to allow the plants to acclimatise slowly to the environment. Once the plants were hardened, the polythene bags were removed completely.

Each treatment had 4 replicates, each containing 4 explants (4 explants × 4 replicates = 16 explants), and repeated twice. All developing buds with laminal shoots were counted as bulblets and the rest of them were counted as bulblet buds at the time of recording data, which was taken after 8 weeks of culture, or as described in the suitable section. The data were subjected to GLM univariate analysis using IBM SPSS 20.0 for windows statistical software. Arcsine transformation was performed for all data in percentages (Snedecor and Cochran, 1967) before subjecting them to the statistical analysis. Post-hoc tests were defined at $P \leq 0.05$ using appropriate methods as described in the footnotes at the bottom of each table.

Results

Bulblet Initiation and Multiplication Using Twin Scale Explants in MS Medium Containing TDZ-NAA

In vitro bulblet regeneration was noted on MS medium containing different concentrations of TDZ-NAA. The data was noted after eight weeks of culture, when most of the explants had stopped regenerating new bulblet buds. The evaluations of the results showed that the percentage of bulb buds per explant induction ranged from 8.33 ± 0.013 to 16.67 ± 0.08. These results were not observed in media supplemented with 0.06811 µM TDZ - 2.685 µM NAA, 0.0908 µM TDZ - 5.37 µM NAA, 0.0908 µM TDZ - 10.74 µM NAA and control MS medium (Table 1). Maximum number of 3.83 ±0.01 bulb buds were noted on MS medium containing 0.0908 µM TDZ - 2.685 µM NAA. Percentage of bulblet regeneration ranged from 50.00 ± 1.89 to 100.00 ± 0.00. Maximum bulblet regeneration was noted on MS medium containing 0.0454 µM TDZ - 10.74 µM NAA, 0.06811 µM TDZ - 5.37 µM NAA and MS medium (control). Maximum number of 8.25 ± 0.05 bulblets per explant were noted on MS medium containing 0.0454 µM TDZ - 10.74 µM NAA (Figure 1a). It was followed closely by 8.00 ± 0.04 bulblets per explant on 0.06811 µM TDZ - 2.685 µM NAA. However, single bulblets were induced on MS medium (control). Bulblet diameter ranged from 0.10 ± 0.001 to 0.18 ± 0.006 cm. The largest bulbs were noted on MS medium containing 0.0454 µM TDZ -10.74 µM NAA.

Table 1 Effects of various concentrations of TDZ-NAA on bulblet regeneration from twin scale explants of *M.neglectum.*

Treatments		Percentage (%) of bulb buds induction*	Mean number of bulb buds per explant*	Percentage (%) of bulblet regeneration*	Mean number of bulblets per explant*	Mean Number of bulblets bulblet diameter (cm)^{ns}
TDZ (µM)	NAA (µM)					
0.0454	2.685	16.67±0.08a	1.15±0.01b	83.33±2.78c	6.00±0.09b	0.13±0.004
0.0454	5.370	0.00±0.00c	0.00±0.00d	75.00± 3.12d	4.50±0.03c	0.15±0.005
0.0454	10.740	8.33±0.013b	0.58±0.06c	100.00± 0.00a	8.25±0.05a	0.18±0.006
0.06811	2.685	0.00±0.00c	0.00±0.00d	50.00±1.89e	8.00±0.04a	0.10±0.001
0.06811	5.370	16.67±0.07a	1.18±0.05b	100.00±0.00a	2.50±0.06d	0.13±0.005
0.06811	10.740	16.67±0.06a	1.25±0.08b	91.67±4.39ab	2.25±0.02d	0.10±0.006
0.0908	2.685	16.67±0.04a	3.83±0.01a	83.33±2.13c	2.00±0.04d	0.11±0.002
0.0908	5.370	0.00±0.00c	0.00±0.00d	75.00 ± 3.77d	6.00±0.07b	0.14±0.007
0.0908	10.740	0.00±0.00c	0.00±0.00d	75.00±4.79d	5.42±0.06b	0.11±0.005
Control (MS Medium)		0.00±0.00c	0.00±0.00d	100.00±0.00a	1.00±0.01e	0.10±0.002

^{ns} nonsignificant, , ± standard error, * Means of values followed by different small letters are statistically different as calculated by Tukeys test at 0.05 level of significance.

Table 2 Effects of various concentrations of TDZ-NAA on bulblet regeneration from bulblets regenerated on twin scale explants of *M. Neglectum.*

Treatments		Initial diameter of bulblets (cm)*	Final diameter of bulblets (cm)*	Diffference in the bulblet diameters (cm)[ns]	Axillary bulblet regeneration percentage (%)*	Mean number of axillary bulblets per explant*
TDZ (µM)	NAA (µM)					
0.0454	2.685	0.29±0.03ab	0.37±0.33ab	0.08±0.004	75.00±3.97bc	1.25±0.04d
0.0454	5.370	0.23±0.03ab	0.35±0.09ab	0.12±0.008	91.67±4.38ab	5.25±0.06ab
0.0454	10.740	0.23±0.07ab	0.25±0.09b	0.02±0.005	100.00±0.00a	6.11±0.05a
0.06811	2.685	0.24±0.01ab	0.48±0.04a	0.23±0.003	66.67±3.73c	1.50±0.5d
0.06811	5.370	0.25±0.02ab	0.44±0.03ab	0.19±0.004	83.33±4.36b	3.23±0.4c
0.06811	10.740	0.25±0.06ab	0.48±0.03a	0.23±0.008	75.00±5.13bc	2.21±0.06d
0.0908	2.685	0.28±0.3ab	0.45±0.06ab	0.17±0.007	75.00±2.99bc	4.75±0.08b
0.0908	5.370	0.31±0.08ab	0.39±0.02ab	0.08±0.009	91.67±3.21ab	5.00±0.09ab
0.0908	10.740	0.15±0.09b	0.31±0.01ab	0.16±0.002	50.00±2.96d	1.25±0.04d

Treatments		Axillary bulblet diameter (cm) [ns]	Mean number of roots per explant (%)*	Mean number of roots per explant [ns]	Mean root length (cm)*
TDZ (µM)	NAA (µM)				
0.0454	2.685	0.13±0.004	83.33±2.89b	2.75±0.09	0.74±0.002ab
0.0454	5.370	0.10±0.009	83.33±3.62b	3.00±0.02	0.87±0.009ab
0.0454	10.740	0.20±0.003	66.67±4.49d	4.38±0.06	0.75±0.002ab
0.06811	2.685	0.10±0.004	91.67±2.10ab	5.25±0.04	1.62±0.008a
0.06811	5.370	0.10±0.009	75.00±5.18bc	2.25±0.06	0.85±0.009ab
0.06811	10.740	0.10±0.004	100.00±0.00a	2.75±0.02	0.57±0.009ab
0.0908	2.685	0.23±0.008	41.67±2.87e	1.53±0.02	0.83±0.001ab
0.0908	5.370	0.17±0.009	91.67±4.00ab	4.58±0.04	1.07±0.002ab
0.0908	10.740	0.10±0.005	66.67±5.40d	2.50±0.01	0.31±0.002b

[ns] non significant, ± standard error, * Means of values followed by different small letters are statistically different as calculated by Tukeys'b test at 0.05 level of significance.

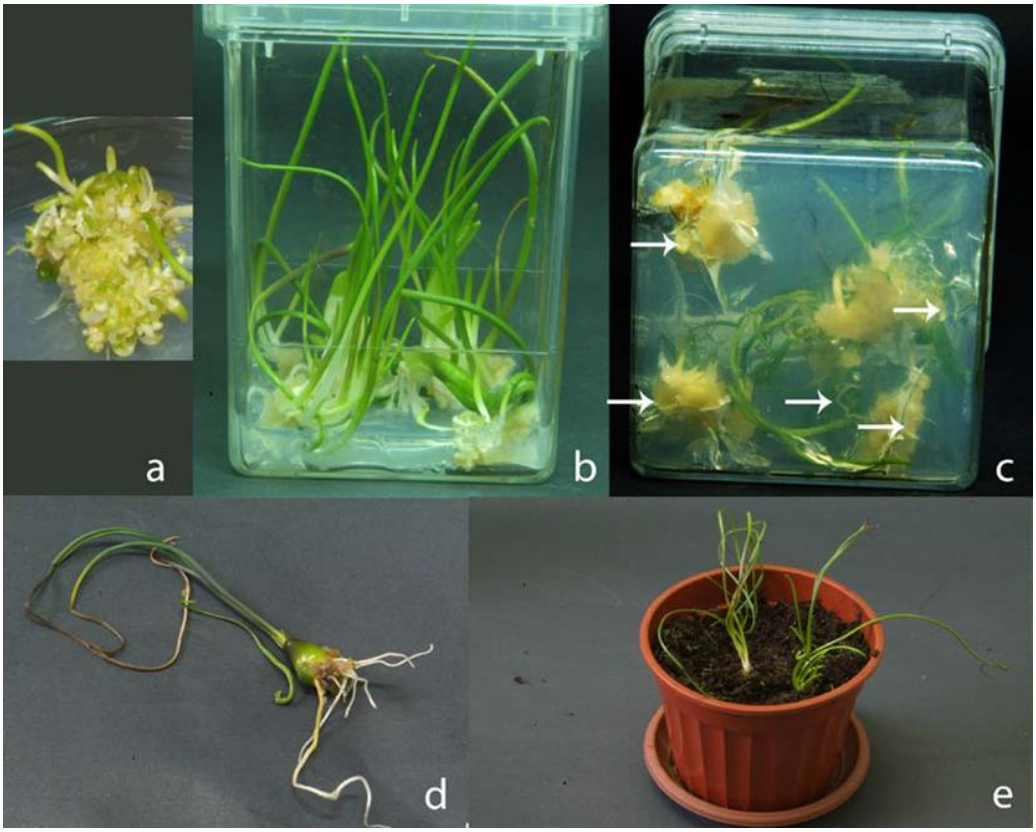

Figure 1 Effects of various concentrations of TDZ-NAA on bulblet regeneration from twin scale explants of *M.neglectum.* (a) Regeneration of bulblets after eight weeks on MS medium containing 0.0454 µM TDZ - 10.74 µM NAA. (b-c) Regeneration on MS medium containing 0.0454 µM TDZ - 10.74 µM NAA showing regeneration of axillary bulblets and (d) roots. (e) Potting of rooted bulblets for acclimatisation.

Effect of TDZ-NAA Concentrations on Clonal Propagation of in vitro Regenerated

Bulblets Used as Explants

The largest bulblets in the range of 0.15 ± 0.09 to 0.31 ± 0.08 cm were subcultured on MS medium containing different concentrations of TDZ-NAA (Table 2). They showed a variable increase in bulb diameters in the range of 0.25 ± 0.09 to 0.48 ± 0.04 cm after another 8 weeks of culture. A comparison of initial and final bulb diameter noted a non-significant increase in their diameters in the range of 0.02 to 0.23 cm. Maximum diameter of the bulblets was noted on MS medium containing 0.06811 µM TDZ - 19.74 µM NAA (Figure 1d).

Axillary bulblet regeneration was noted on all mother bulblets in the range of 50.00 ± 2.96 to 100.00 ± 0.00. The number of axillary bulbs ranged from 1.25 ± 0.04 to 6.11 ± 0.05 bulblets per mother explant. Maximum number of axillary bulblets were noted on MS medium containing 0.0454 µM TDZ - 10.74 µM NAA (Figure 1b and c). The bulblet diameters increased in the range of 0.10 ± 0.009 to 0.23 ± 0.008 cm. After 2-3 weeks of subculture, the mother bulbs began to develop root initials, which developed later into full length main roots. Mean rooting percentage ranged from 41.67 ± 2.87 to 100.00 ± 0.00. All bulblets showed rooting on MS medium containing 0.06811 µM TDZ - 10.74 µM NAA. The number of roots per mother explant and mean root length varied in the range of 1.53 ± 0.02 to 5.25 ± 0.04 and 0.31 ± 0.002 to 1.62 ± 0.008 cm, respectively. The bulblets attained sufficient diameter and roots; therefore, they were not further subcultured to improve rooting.

Acclimatization of TDZ-NAA Regenerated Bulblets

The acclimatized bulblets were transferred to pots containing compost, where these bulblets developed green leaves and continued the growth after acclimatisation, and were uprooted after eight weeks. Examination of the acclimatized bulblets after two months showed the replacement of *in vitro* regenerated roots by new thin and branched root apparatus with a reduction in the size of the potted bulblets. The bulblets took a period of about two weeks to acclimatize depending on the health and vegetative maturity of them, and the environmental conditions. All bublets regenerated on TDZ- NAA could be acclimatized (Figure 1 e).

Discussion

The species belonging to Muscari genus are famous for their uses as ornamental plants all over the world. Most of these are yet to be exploited for *in vitro* or *ex vitro* cultures under field conditions. Biotechnological approaches could serve as an easy alternative to the conservation of local germplasm in case there is an availability of a suitable protocol that could allow clonal propagation. The current study presents an efficient *in vitro* clonal propagation system of *M. neglectum* on MS medium containing variable concentrations of TDZ-NAA.

Bulblet Regeneration Behaviour

The *twin scale explants* behaved variably on MS medium containing different concentrations of TDZ-NAA. Comparison of regeneration on *twin scale explants* showed the maximum number of 8.25 ± 0.05 bulblets per explant on MS medium containing 0.0454 µM TDZ – 10.74 µM NAA with a maximum bulblet diameter of 0.18 ± 0.006 cm. The results of the study suggest that the indvidual regeneration on TDZ-NAA-containing MS medium was strongly influenced by the concentrations and combinations of plant growth regulators. The results are in agreement with Uranbey (2010 a, b), Uranbey et al. (2011), Ozel et al (2007, 2009, 2015), Vaziri et al. (2014) and Uzun et al. (2014), who reported the propagation of other Turkish *Muscari* species using different cytokinins and auxin concentrations.

The regeneration was directly related to the number of induced bulblet buds per explant. The results are in agreement with Hutchinson et al. (2004), who suggested a synergistic effect between –cytokinins and auxins in induction of shoots, roots and bulbsusing shoot tip explants of *Ornithogalum saundersiae*. Malabadi and Van Staden (2004), Mirici et al. (2005), Suh et al. (2005) and Uzun et al. (2014) also suggested beneficial effects of TDZ usage in combination with auxin(s) in regeneration of bulbous geophytes. Results of all studies suggested that type of explant has clear bearing and influence on the rate of regeneration and multiplication of the species that is micro propagated under *in vitro* conditions besides the plant growth medium, and the growth regulators used in the study.

Bulblet Diameter

The largest bulblets of 0.18 ± 0.006 cm were recorded on MS medium containing 0.0454 µM TDZ-10.74 µM NAA; however, when they were subcultured, the maximum increase in bulblet diameter on TDZ-NAA containing medium showed bulblets of similar size. TDZ-NAA had more synergistic effects on bulblet diameters. Moreover, TDZ-NAA were not toxic for *M. neglectum* without any carry over effect. High activity of TDZ-NAA has not been investigated in *M. neglectum* at the molecular level. It is assumed that TDZ-NAA has a synergic effect in a manner similar to Gill and Saxena's study (1992). They have suggested a crucial role of TDZ in the interaction with endogenous plant hormones that reprogram the mode of morphogenesis. This possibly acts by releasing, synthesising, protecting or even inhibiting auxins *in situ* in combination with other sub-cellular metabolic changes, in regulatory enzymes and related proteins. All (100%) bulbs induced under *in vitro* conditions were easily acclimatised. However Uranbey et al. (2011) used 5-10 mm bulblets of *M. azureum* and acclimatised them on compost with a survival rate of 3%. The possible reason could be different type of materials used for rooting. This experiment induced rooting under controlled *in vitro* conditions. Whereas, Uranbey et al. (2011) used compost instead to root the *Muscari* bulbs. It seems as if the Nitrogen rich compost had negative effect on the bulbs that most often results in burning of the plant material.

Rooting

Comparing different concentrations of TDZ-NAA in MS medium on induction of the roots, a negligible carry over effect of these concentrations was noted. All of the induced bulblets did not need a separate rooting medium. The bulblets behaved variably in terms of root mean number and root length. It was also noted that MS medium (control) used singly was ineffective to induce roots. These results are not in agreement with Uranbey (2010a,b), Uranbey et al. (2010), Vaziri et al. (2014), Ozel et al. (2007, 2009, 2015) and Uzun et al (2014)'s studies. The authors used either NAA or IBA for the rooting of different species of *Muscari*. These results are in agreement with the results reported by Ozel et al. (2009), who had almost similar observation on rooting of *Muscari macrocarpum.*

Number of Roots

Considering the number of roots per explant and their length on the bulblets regenerated on MS medium containing different concentrations of TDZ-NAA, the longest and maximum number of roots were noted on 0.0454 µM TDZ – 10.74 µM NAA containing MS medium. These bulblets were acclimatized in the growth chamber. It is assumed that synergetic effect of TDZ-NAA in interaction with endogenous plant hormones and other sub-cellular metabolic changes played a key role in the elongation of roots in agreement with the observations of Alizadeh et al. (2013) in *Allium tuberosum.*

Acclimization

The acclimatized bulblets were transferred to pots containing leaf compost and were uprooted after eight weeks. The initial fragile thick root structure of the acclimatized bulblets was replaced by much more supportive longer and multi-branched secondary roots which is in agreement with resulted of Ozel and Khawar (2007) at the end of the experiment. Khawar et al. (2005) and Ozel et al. (2009) emphasize that transplantation of bulblets from *in vitro* to *ex vitro* conditions needs some period for acclimatization that could end up with variable number of survival of *in vitro* regenerated bulblets depending on bulblets health, their vegetative maturity and prevailing environmental conditions. Ozel et al. (2009) acclimatized *M. macrocapum* bulblets in the growth chamber at constant temperature of 24 ± 1°C. Similarly, Wang et al. (2013) acclimatized *M. armeniacum* on perlite and torf (1:3) mixture for 4 weeks. On the other hand, Uzun et al (2014) induced 100% acclimatization of *Muscari muscarimi* in peat moss, vermiculite and perlite (1:1:1) mixture. Uranbey et al. (2011) used 5-10 mm bulblets of *M. azureum* and acclimatized them on compost with a survival rate of 3%. The possible reason for this observation could be the difference in genotypes, cultural conditions and type of composts that were used in two experiments during acclimatisation.

Regeneration of New Roots

New roots were induced on all bulbs after they were transferred to soil. Replacement of *in vitro* regenerated roots by new thin and branched roots in the soil is a positive factor suggesting that the bulblets could induce root initials in the soil, once they find appropriate environment. This suggests that the bulblets need an appropriate vegetative growth stage to induce roots in the soil. Similar observations were recorded in *Ornithogalum oligophyllum* (Ozel and Khawar, 2007) and *Muscari macrocarpum* (Ozel et al., 2009).

No problem was noted in the acclimatisation in compliance to the findings of O'Connor et al. (2007), who observed that the explants with high number of short and stout roots were more likely to survive acclimatisation. They suggested that small, healthy and strong rooted bulblets were easily acclimatised with minimum damage to roots.

Conclusion

This study provided an important addition to the literature on microclonal propagation of *M. neglectum*. Use of auxins for root induction was not needed separately. TDZ induced faster induction of physiologic age on the bulblets that influenced rooting positively. It meets the objective of the study and provides a good information about the potential growth regulator combinations for bulblet regeneration from twin scale explants of *M. Neglectum.* The results suggest potentiality of this protocol for commercial propagation.

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