Improved micropropagation of biopesticidal plant, *Pelargonium radula* via direct shoot regeneration

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Abstract

This review highlights the recent development and achievements made for the rapid and simple micropropagation of *Pelargonium radula* in Malaysia. Microshoots were regenerated from nodal explants of stem through auxiliary shoot proliferation. The proliferatoin of multiple shoots from nodal segments was the highest in MS medium supplemented with 0.5 mg/l BAP+1.0 mg/l IBA. For rooting different concentration of IBA were used and highest rooting was recorded on MS medium with 0.2 mg/l IBA+0.2 mg/L IAA. The rooted Plantlets were direct transferred into the pot with medium organic soil and garden soil given the highest in survival rate up to 96%. The process carried out directly at the glasshouse without going through the process of acclimatization. The use of un-sterilized mix rooting media makes the process easier to handle.

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Introduction

Scented pelargoniums are great garden plants. Not only do they produce colourful flowers, aromatic smells and interesting leaf shapes, they have many uses. Some can be used in cooking, others for pest control and some for potpourri. *Pelargonium radula* is a species in the Pelargonium genus, originally Malaysian's plant. Its general name is geranium but Malaysian calls it as Jeremin. Our ancestors call it as "pokok halau nyamuk" as it was used as mosquitoes repellent or can be used as a biopestidal plant and will contribute the greener world. *Pelargonium* spp. or geranium essential oil is a plant extract used as the active ingredient in insect repellent formulation. Another name for *Pelargonium radula* is *Pelargonium graveolens*. *Pelargonium radula* produces a characteristic essential oil having terpene alcohols as major components, e.g geraniol, cintronellol and their esters, e.g. i-methnone, citronellyl formate, and geranyl formate also eugenol (Kalodjer et al.2001). These components are believed to have potential insect repellence activity (James, 2003). Linalool is major biopesticide chemical compounds found in *Pelargonium radula*. Linalool is found naturally in a variety of plants, flowers and spices. As a pesticide, linalool is intended for use indoors to control pests (fleas and ticks) on pets and the spaces they inhabit by affecting the insect's nervous system. Linalool is also used as an outdoor mosquito inhibitor. Because of its

flavourful and fragrant properties, linalool has non-pesticide uses and it is added to processed food and beverages, perfumes, cosmetics and soaps as well as to household detergents and waxes.

Pelargonium spp. be able to live Malaysia's climate but cannot directly expose towards sunlight. In Malaysia, the plant is widely planted in Cameron highland and can be easily found at nursery nationwide. However, sufficient number of plant materials is often a problem in the plantation sector. Sufficient numbers of plant material are also needed to be propagated for newly introduced varieties. In the view of potential commercial value, it is highly desirable to develop a simple rapid, efficient and large scale propagation of *Pelargonium radula* through tissue culture.

Material and Methods

Plant material and aseptic culture establishment

The healthy and mature of biopesticidal plants, Pelargonium radula having an age of 1-2 months-old was maintained in glass house for a few weeks prior to explant excision and establishment in vitro. After defoliation the leaves and segment of plants collected then were washed in running tap water for 1 hrs. The explants were cut into pieces of about 3-5 cm, divided into four different segment, petiole, stem (branches/ trunk), side shoot (lateral) and shoot apex. The segment kept in a conical flask and washed with detergent (Teepol) solution for 20 mins followed by rinsing in distilled water. The explants were transferred to laminar air flow chamber and were finally surface sterilized with 10-20% of Clorox® containing several drops of Tween-20 for 5-20 mins on a rotary shaker. After three rinses with sterile water and removal of the damaged ends, the different segment of explants were cut into small pieces then cultured on MS medium treatment. Cultures were checked regularly for contaminations and those presented apparent infection symptoms were immediately discarded.

Initiation/establishment and micropropagation of shoot cultures

Sterilized segment of the explants were cultured individually under aseptic conditions on basal MS medium containing 3.0% sucrose and 0.3% gelrite agar for gelling. Depending on the experiments, for initiation the MS medium was also supplemented with BAP or IBA in four different concentrations (0, 1.0, 3.0, or 5.0 mg/L), respectively to induce shoot formation. Sterilization was performed by autoclaving at 121 ^{o}C for 20 min. pH was adjusted at at 5.7-5.8 before adding agar. Explants were inoculated on 150 ml flask containing 40mL of the desired medium. Ten replicated flasks were used in each medium treatment. Each flask contained 3 explants which were contact with the medium surface. The cultures were incubated in a plant growth room at a temperature of 25 ^{o}C±1 and with 16 h photoperiod by cool-white fluorescent lamps (1000–2000 lux). The comparative increment in the shoot multiplication for micropropagation, separate experiment was conducted. Established in vitro explants were cultured on basal MS medium supplemented with BAP (0, 0.5, 1.0, and 3.0) in combination with either IAA, GA_3 or IBA at concentration 0.2, 0.5 and 1.0 mg/L, respectively. Observations were recorded at weekly intervals. Results were expressed as number of shoots per culture and length of the shoots after 45 days of culture. Standard error of the mean was calculated for the degree of response which is represented in Table as ±value.

Rooting of shoots and transplantation of plantlets

Shoots (>2.0 cm long) from the best treatment and obtained at the end of the elongation stage with at least four leaves were individually placed inside flaks. The explants were cultured on MS medium supplemented with either IBA (0, 0.1, 0.2, 0.5, 1.0, 1.5, 2.0 mg/L), IAA (0, 0.1, 0.2, 0.5, 1.0, 1.5, 2.0 mg/L) or combination of both. Ten replicated flasks were used in each medium treatment. The number of shoots producing roots, as well as the number and length of the induced roots were recorded after 30 days. *In vitro* raised plantlets were removed from the culture medium and roots were washed under running tap water to remove agar. They were then transferred in 15 cm pots to greenhouse under shade. The pot containing different type of medium was used as treatments; vermiculite mixed with sterile garden soil (1:1), organic soil mixed with garden soil (1:1), sand mixed with garden soil(1:1), vermiculite mixed with non-sterile garden soil (1:1), organic soil mixed with non- sterile garden soil (1:1) and sand mixed with non-sterile garden soil (1:1). Well-established plantlets were finally transplanted to the field. Observations were recorded with respect to the percent survival of rooted and acclimatized plants. Standard error of the mean was calculated for the degree of response which is represented in Table as ±value.

Result and discussion

Establishment of shoot regeneration

A simple and effective protocol has been developed for the in vitro plant regeneration and micropropagation of *P. radula*. We investigated the effect of plant growth regulator, cytokinins and auxins on the efficiency of shoot organogenesis. Different of explants were cultured on MS media supplemented with different concentration of BAP and IBA to evaluate their effect on *P. radula* shoot multiplication. For the duration of 40 days, on all tested media, shoots developed directly from meristematic explants (shoot apex and side shoots) and indirectly through redifferentiation of callus, which often was induced at the base of the petiole and stem. In general, MS medium supplemented with BAP was more effective in promoting shoot than those supplemented with IBA. It has been emphasized by Adel and Sawy (2007) about the importance of BAP for regeneration of in vitro plant. According to Tavares et al. (1996), increased of BAP concentration up to 1 mgl/l showed greatest efficiency in shoot number of *Melissa spp.* Faisal et al.(2010) reported that range of 1-2 mg/L BAP gave the best result in terms of maximum number of shoots per explant. On the media containing BAP, all explants showed shoot initiated up to 9 shoots per explants during 40 days. (Table 1). This fact can be explained that cytokinins especially at the high concentration overcome apical dominance and promote shoot formation (cheverrigary and Fracaro, 2001). Explants grown on PGR-containing media presented different number of shoot initiation depending on the type of explants cultured. Among the different explants cultured, stem performed the best on shoot initiation. Observation showed that with the 3mg/L BAP produced the highest number of shoot(9) from stem explants, however the shoot length only at 2.1cm. With the stem explants, growth was initiated by the enlargement of the explants followed by the production of small and round globules along the cuts across the mid-vein. These globular structures, however, were able to emergence, and convert into axillary buds

then develop further to multiple shoots (Figure 1(a-b). Results also indicated that addition of IBA induced high elongated shoots up to 4.1 cm, although more shoots proliferated on initiation medium supplemented with BAP. At 3 mg/L BAP, lower in elongated shoots were obtained in all explants tested compared to those in IBA. Table 1 clearly indicates that shoot proliferation was lower at low concentrations of BAP and gradually increased with the increase in BAP concentration. It became maximum at 3 mg/L BAP, but again decreased as BAP concentration at 5 mg/L was applied. It was generally recognized that concentration of cytokinin in the medium should be optimum for getting increased shoot proliferation of plants (Sharp *et al*., 1984).

Table 1: Effect of different concentrations of BAP or IBA on shoot initiation from different segment of explants after 40 days of cultured.

Explants segment	Growth regulators Cons.	No.of shoot/explants	Average length of shoot(cm)
Shoot apex	BAP		
	0	1±0.23	0.2
	1	3±0.24	2.9
	3	2±0.50	2.7
	5	1±0.36	0.9
	IBA		
	1	2±0.12	4.1
	3	1±0.23	3.1
	5	1±0.45	2.3
Stem	BAP		
	0	0	-
	1	7±1.21	2.9
	3	9±2.10	2.1
	5	3±0.91	1.5
	IBA		
	1	3±0.12	3.9
	3	2±0.23	3.7
	5	2±0.12	2.8
Side shoots	BAP		
	0	2±0.21	3.1
	1	4±0.41	2.4
	3	6±0.34	1.5
	5	5±0.11	1.2
	IBA		
	1	2±0.12	3.3
	3	1±0.05	3.5
	5	2±0.34	1.2
Petiole	BAP		
	0	0	-
	1	1±	0.9
	3	2±	0.8
	5	0	-
	IBA		
	1	0	-
	3	0	-
	5	0	-

Shoot multiplication

The effects of different concentration concentration of BAP on shoot multiplication either with or without various plant growth regulators (IAA, GA3 and IBA) were investigated. After the initial shoot from stem explants was subcultured into different combination of hormone, multiple adventitious shoots were produced from the base of the explants after 30 days. Table 2 shows the percentage of shoot proliferation, number of shoots/explants and the length of shoots obtained on media tested. Different concentrations and combinations of growth regulators showed different responses. In general, MS medium supplemented with BAP combined with IBA or GA3 were more effective in promoting shoot development than those combined with IAA. This study had shown that increasing the concentration up to 3mg/L of BAP during the initiation stage enhanced the number of shoot production. The importance of the application of high BAP concentration to initiate shoots formation from explants were reported by Zaffari et al. (2000). Upon subculture for shoot proliferation, the percentage of shoot proliferation and number of shoots/explants was highest when 0.5 mg/L BAP in combination with 1 mg/L IBA was used with resulted at 95 % and 22 shoots/explants, respectively. When IBA was added to the medium at all concentration, it appears that shoot production had a tendency to decrease as BAP concentrations increased considerably. Shoot proliferation were seriously reduced by combinations of high BAP (1.0 or 3.0 mg/l) and all concentrations of IBA (0.2, 0.5 and 1 mg/l). In addition, lower BAP concentrations (0.5 mg/L BAP) with combination of IBA become more favourable for shoot proliferation. Presence of IBA promotes shoot proliferation and elongation as reported by Najmeh et al. (2011). Generally, the results have shown that shoot multiplication and elongation was significantly better in the presence of IBA than BAP alone. Dhed'a et al. (1991) reported that combinations of BAP with IBA were effective for in vitro multiplication of plantlets of banana. As for shoot elongation, the best result was obtained when 1 mg/L of IBA alone was applied with average 6.1 cm. Shorter shoots developed at higher concentrations of BAP (3 mg/L). Najmeh et al. (2011) reported that application of high concentrations of BAP after shoots initiation was not essential for shoot propagation due to the reduction in the number of shoots and high incidences of abnormality. Addition, high concentrations of BAP did not allow recovery of the explants in tissue cultures in becoming complete normal plants due to the habituation effect of BAP.

Generally, MS media supplemented BAP in combination with IAA did not show better response in shoot development as IBA or GA3. By adding IAA into the media combined with BAP resulted lower in shoot proliferation. However, Resmi and Nair (2007) reported, high shoot multiplication but a reduction in the length of shoots in media with a combination of BAP and IAA in triploid cultivar. Most of the combinations of BAP with IAA showed good response shoots elongation, which maximum number of leaves was observed in combinations of 1.5 mg/L BAP and 0.5 mg/L IAA (Thangapandian et al. 2012). The best response to shoot regeneration in *M. officinalis* were also obtained on medium supplemented with IAA and BA (Gbolade and Lockwood, 1992). When GA3 was included into media with combination of BAP seemed to broadly promote shoot proliferation. The

best shoot proliferation is when being treated with combination of 0.5 mg/L BAP and 0.5 mg/L GA3 with where percentage of explants showing shoot proliferation recorded 86 % with shoot number of 21 and average shoot length of 4.4 cm. In contras Ihsan et al (1995) stated that GA3 and 0.5-1 mg/L, a fairly good amount of multiplication shoot were formed but shoot tended to elongate rather than to multiply. According to Sultana et al (2012), elongation of shoots was found to be most excellent in medium containing GA3 over other media supplemented with different hormones. Applied with GA3 (2.89 mM into medium producing maximum elongation of Mucuna prureins (Sathyanarayana *et al.* 2008). The gibberellins are concerned in several physiological process regulations such as seed germination, initiation and growth of flowers and shoot elongation (Rkhis *et al.* 2006) observed that GA3 (gibberellic acid) has role in cell elongation in stems.

Table 2: The effects of different concentration concentration of BAP combination with various plant growth regulators (IAA, GA3 and IBA) on shoot proliferation, nember of shoots/explants and average length of shoots.

BAP (mg/L)	IAA (mg/L)	% of explants showing shoot proliferation	No.of shoots/explants (mean±SD)	Averange length of shoots(cm)
0	0	25	4±0.24	4
	0.2	32	6±0.15	2.5
	0.5	50	8±0.43	1.9
	1.0	48	4±0.12	2.4
0.5	0	50	7±0.42	4
	0.2	65	8±0.31	3.2
	0.5	74	8±0.54	3.9
	1.0	61	5±0.52	2.1
1.0	0	76	8±0.71	2.5
	0.2	64	7±0.32	3.2
	0.5	62	7±0.24	2.8
	1.0	52	5±0.12	2.9
3	0	68	5±0.45	1.9
	0.2	52	5±0.13	1.8
	0.5	61	6±0.21	1.6
	1.0	42	3±0.14	1.5
BAP (mg/L)	GA_3 (mg/L)			
0	0.2	74	9±1.32	3.9
	0.5	81	10±1.24	4.4
	1.0	84	13±2.13	5.9
0.5	0.2	82	17±1.41	3.1
	0.5	86	21±2.13	4.4
	1.0	83	16±1.92	4.2
1.0	0.2	71	7±1.41	2.9
	0.5	84	11±0.92	2.8
	1.0	89	12±1.31	3.1
3	0.2	64	8±1.11	1.9
	0.5	58	7±0.64	2.4
	1.0	52	7±0.23	3.6
BAP	IBA			

(mg/L)	(mg/L)			
0	0.2	74	14±2.41	3.4
	0.5	81	18±1.61	5.8
	1.0	81	17±4.51	6.1
0.5	0.2	89	17±3.21	4.1
	0.5	87	16±2.23	4.2
	1.0	95	22±4.12	4.9
1.0	0.2	74	16±3.12	2.4
	0.5	72	15±2.52	2.5
	1.0	65	13±1.34	1.9
3	0.2	64	11±1.34	1.4
	0.5	60	11±2.11	2.1
	1.0	55	9±0.96	1.9

Rooting and acclimatization

Generally, all treatment showed response on percentage of explants rooted. Significant differences were noted between the treatments for rooting. Results in (Table 3) show that the treatment of 0.2mg/L IBA combined with 0.2 mg/L IAA was the highest percentage of rooted followed by 1 mg/L IAA which produced 96 and 95 percent rooted, respectively. Comparatively, the highest number of roots was observed at 0.2 mg/L IAA alone or combination with 0.2 IBA at 13 roots/explants after 30 days of culture, respectively. It was followed by 0.5 mg/L IAA alone or combined with 0.2 mg/L IBA resulted 11 roots/explants, respectively. Regarding root length, a similar trend was observed with the highest mean root length observed at o.2 mg/L IAA (3.5 cm) followed by 0.5 IAA (3.1 cm) and 0.2 mg/L IBA combined with 1 mg/L IAA (2.5 cm)). Maximum and minimum level of IBA produced poor results than the optimum levels. The results show that the effect of IAA and IBA concentrations was found significant for root formation.

With respect to number of root produced per explants under various plant growth regulator treatments, the MS medium supplemented with IAA was superior compare to IBA. Newest reported by Nasser et al. (2012), in most of the lemon balm spp, maximum root percentage and growth was observed in medium treated with 2 mg/L IAA . Silva et al.(2005) using in vitro of Melissa officinalis under growth regulators obtained maximum root production in the medium supplemented with IAA. The effect of auxins on root formation has been well documented in several plant species (Blakesley and Constantine, 1992; Fracaro and Echeverrigary, 2001.) Auxins alone or in combination with very low concentration of cytokinins were effective in induction of root primordia (Pierik, 1997). Liu et al. (2002) reported that auxin (IBA and NAA) induces the complicated process of lateral root formation through repetitive cell division. George et al. (2008) suggested that auxins are essential for the maintenance of polarity of the plants. In addition, high of IBA reported to have a reduced number and length of roots. Moreover, higher levels of IBA applied to plants inhibit the formation of shoot buds and this might further stop the production of roots as the auxin in the root primordial is shifted from the shoot apex (Ozel et al. 2006).

Table 3: Effect of different concentration of IBAand IAA on % of explants rooted, no. of roots per plant and average root length of *Pelargonium radula*

IBA (mg/L)	IAA (mg/L)	% of explants rooted	No.of root/explant	Average root length (cm)
0	0	65	5±0.39	1.9
0.1	0	67	7±0.91	2.2
0.2	0	69	8±0.72	2.0
0.5	0	54	7±1.09	1.6
1.0	0	63	5±0.34	1.2
1.5	0	61	5±0.51	1.4
2.0	0	54	4±0.36	1.3
0	0.1	74	8±0.94	2.1
0	0.2	81	13±1.34	3.5
0	0.5	85	11±1.04	3.1
0	1.0	95	9±1.61	2.3
0	1.5	83	8±0.71	2.4
0	2.0	81	5±0.24	1.9
0.2	0.2	96	13±0.92	1.3
0.2	0.5	84	11±0.53	1.9
0.2	1	89	8±0.72	2.5
0.5	0.2	85	7±0.81	1.7
0.5	0.5	84	6±0.62	1.8
0.5	1	81	7±0.53	2.1
1	0.2	70	5±0.31	1.4
1	0.5	71	6±0.42	1.9
1	1	69	8±0.92	1.8

Table 4: Effect of different type of medium on rooting (%) and survival(%) of in vitro *Pelargonium radula* after 60 days of transplanting.

Type of medium	**% rooted**	**% survival**
Sterile medium		
Vermiculate+ garden soil	78	54
Organic soil+garden soil	98	96
Sand+garden soil	81	57
Non-sterile medium		
Vermiculate+ garden soil	76	56
Organic soil+garden soil	98	95
Sand+garden soil	78	67

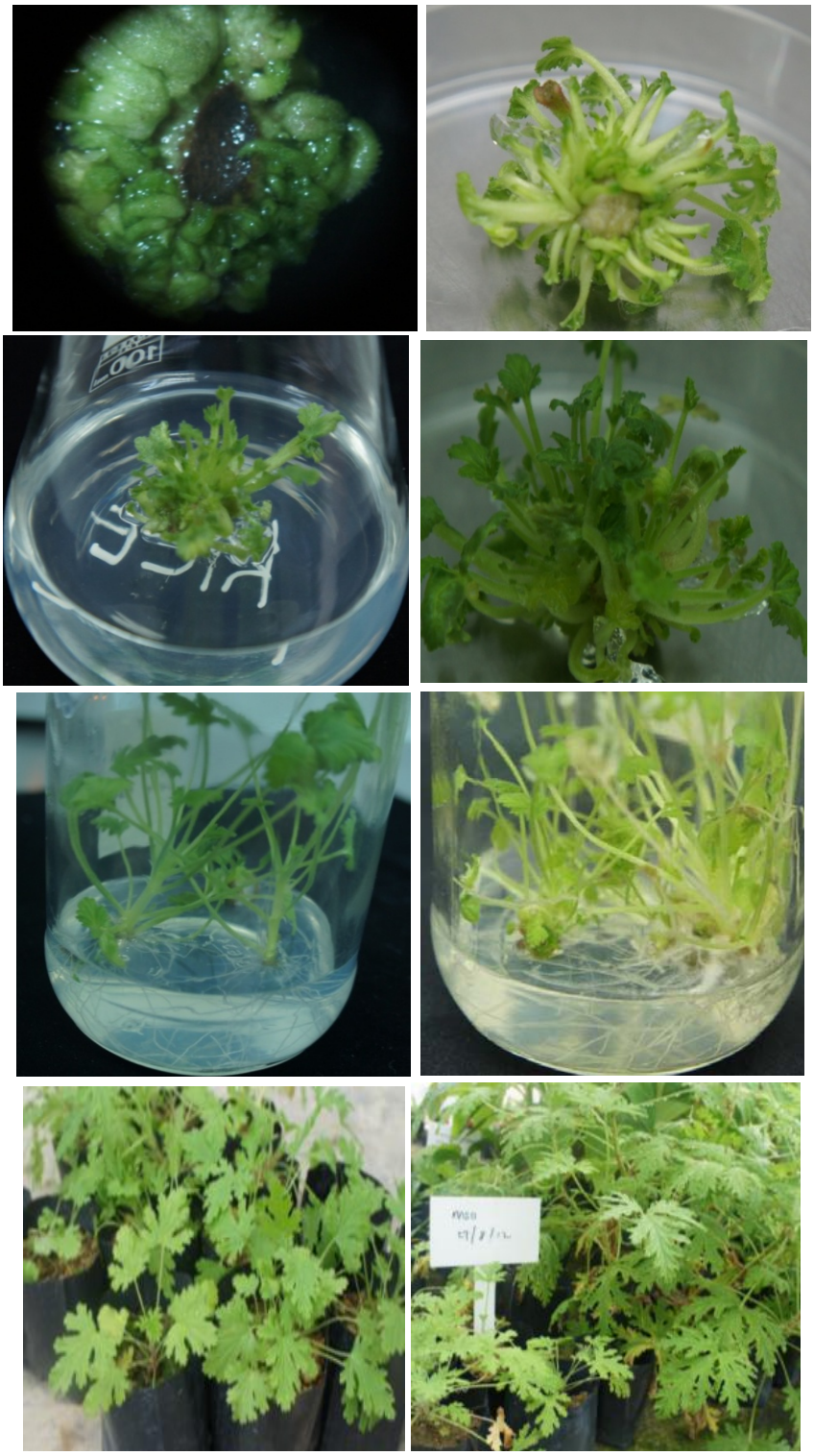

Figure 1: Direct plant regeneration of in vitro cultured *Pelargonium radula* . Early stage of shoots formation from stem on medium with 3.0 mg/1 BAP *(a)* . Development of shoots from stem on medium with 3.0 mg/1 BAP (b). Prolifaration and development of shoots (c) (d) . Root elongation on basal medium (e) and medium with IBA (f). Plantlets transferred into polibag after one month (g) and 3 months (h).

The in vitro rooted plantlets were direct transplanted to pot under greenhouse condition with 70% sun light exposure. There is no acclimatization process were involved in this technique. The pots containing different combination of media were tested for the percentage of rooted and survival. Generally, sterile application of the media did not show any significant changes as compared to those non sterile. After 20 days transferring the rooted explants to the pot, organic soil+garden soil media showed the highest in percentage of rooted up to 98 %. This media also resulted highest in survival rate after 60 days of transplanting with 96%, followed by sand+garden soil media (57-67 %) and vermiculate+garden soil media (54-56%). Plants appeared morphologically uniform with normal leaf form, shape and growth pattern.

Conclusion

Our research shows that the shoot multiplication of common thyme is depended upon the treatment that is used. The highest number of adventive shoots was obtained on the MS media supplemented with 0.5 mg/L BAP and 1.0 mg/L IBA, which employed the highest concentration of cytokinins. Overall finding of the present study are significant in obtaining the maximum regeneration with proper concentration of growth regulator. In conclusion we have developed a promising method for an efficient and simple micropropagation of *Pelargonium radula*. The protocol could be useful for large scale production of a single genotypes like this cultivar. Present results also showed that phenotypically there were no observable variations between the parents and in vitro propagated plants. The plantlets were hardened with a survival rate of up to 98% and then established well after planting them in a glass house.

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