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RESEARCH ARTICLE

ESTABLISHMENT OF ROOT CULTURES IN *Pelargonium Radula*

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ABSTRACT

Root cultures were initiated from stem, petiole and leaf explants of *Pelargonium radula*. Stem segments, the explants most amenable to adventitious root induction, developed well when cultured on half strength solid MS (Murashige & Skoog) medium containing 15 g/L sucrose. To enhance the induction and proliferation of adventitious roots, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) or α-napthalenacetic acid (NAA) were added to the culture medium. Supplementation with 0.5 mg/L NAA gave the highest incidence of root initiation (97.7%) and the highest number of roots (8.1 roots/explant). Root cultures were subsequently established in continuously agitated liquid medium. Up to 17 g/flask fresh weight of roots were obtained in full strength liquid MS medium containing 0.5 mg/L NAA, with the cultures maintained in either light or darkness.

INTRODUCTION

Pelargonium radula (Cav.) L., originating from South Africa, is a member of the Geraniaceae family. The plant, which is known for its hypoglycaemic properties, is grouped in the category of scented geraniums. It produces an essential oil known as jeramin oil that is widely used in the cosmetics and fragrance industry. As an environmentally friendly and biodegradable anti-microbial, it is effective against fifteen species of fungi and fourteen species of bacteria, including *Pseudomonas aeruginosa, Bacillus pumilus, Bacillus subtilis, Escherichia coli* and *Serratia marcescens* (Pepeljnjak *et al*., 2005). *P. radula* extracts possess antioxidant activities and they reduce LDH leakage from Hep G2 cells (Petlevski *et al*., 2013). These extracts may be used in complementary therapy for the treatment of diabetes. *P. radula* also contains geraniol, cintronellol, and esters such as i-methnone, citronellyl formate, geranyl formate, and eugenol. As an essential oil, geraniol is used to treat dysentery, haemorrhoids, inflammation, heavy menstrual flow and even cancer (Lis Balchin 2002, Tajkarimi *et al*., 2010). As in many other medicinal plants, the composition of medicinal compounds and their potency in *P. radula* are influenced by environmental and physiological conditions, especially those that interfere with the stable production of these compounds (Beppu *et al*., 2004). To overcome environmental variables, plant *in vitro* culture is an alternative way to obtain useful secondary metabolites for producing valuable phytochemicals. For example, cultured adventitious root systems have been established for production of anthraquinone in *Morinda citrifolia* (Sato *et al*., 1997; Baque *et al*., 2010), lucidin-3-*O*-primeveroside in *R. tinctorum* (Sato *et al*., 1997) and aloe-emodin in *Aloe vera (*Lee *et al*, 2011). The rapid growth and stable mass production from adventitious root cultures of many plant species offer promise for high productivity of valuable secondary metabolites used as pharmaceuticals, pigments and flavours (Srivastava and Srivastava, 2007; Murthy *et al*., 2008). The current study was conducted to optimize *in vitro* culture conditions for adventitious root cultures derived from *P. radula* stem tissues.

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MATERIALS AND METHODS

Plant material

Mature plants of Pelargonium radula sourced from the Cameron Highlands (Malaysia) were used as starting materials for *in vitro* culture. Segments of stems, petioles and leaf laminae were surface-sterilized by immersion in 10-20% (v/v) sodium hypochlorite (Clorox®) containing several drops of the detergent Tween-20 (Zuraida *et al*, 2013). After 30 min on a rotary shaker, the explants were given three rinses with sterile water and transferred aseptically to basal MS medium (Murashige and Skoog, 1962) containing 3.0% sucrose and 0.3% gelrite agar. The culture medium pH was adjusted to 5.7-5.8 before the addition of the agar. Sterilization of the medium was performed by autoclaving at 121° C for 20 min. *In vitro* regeneration of plantlets was carried out according to the procedure of Zuraida *et al*. (2013) (Figure 1a). The plantlets obtained were grown in a plant growth room at 25±1 ° C under a 16 h photoperiod with lighting provided by cool-white fluorescent lamps (1000–2000 lux). Stems (1.5 cm), leaf petioles (1.5 cm) and leaf lamina pieces (1.0 × 0.5 cm) from six weeks old cultured plantlets were used to determine the best explants for root induction and subsequent growth.

Culture conditions for root proliferation

Segments of stems, petioles and leaf laminae were cultured under aseptic conditions on solid MS media with various combinations of growth regulators. The explants were placed in plastic Petri dishes (ten per dish and ten dishes per treatment) containing 30 ml of solid culture medium containing 0.35% wt/vol Phyto agar. For root induction, the explants were cultured on MS medium containing different concentrations of auxins (IAA, IBA, NAA), ranging from 0.2 to 3.0 mg/L. The percentage root initiation and number of roots for the different explants were recorded after a 45-day culture. To establish the best root proliferation medium, an optimization process was carried out step-wise on 4-week old root cultures of *P. radula* (0.2 g per petri dish) on solid. Culture media containing 0.5 mg/L NAA. The medium variables comprised (a) five different concentrations of MS media, *viz.* MS ¼, (quarter strength), MS ½ (half-strength), MS 1 (full-strength), MS 1 ½ and MS 2, and (b) five sucrose concentrations, *viz.* 1.5 %, 3.0 %, 4.5 %, 6.0 % and 7.5 %. The root cultures were incubated under continuous light or in total darkness for 45 days. After the prescribed culture period, the roots were harvested and their fresh weight recorded. In another set of experiments, root cultures were weighed (0.2 g per flask), and transferred to liquid media containing 3.0 % sucrose and 0.2-1.5 mg/L NAA or 0.2-1.5 mg/L IBA in 250 mL conical flasks. The cultures were kept in light or darkness on an orbital rotary shaker set at 110 rpm to prevent anoxia. To study the effects of different MS concentrations on root proliferation, five MS concentrations, *viz.* MS ¼, MS ½, MS 1, MS 1 ½ and MS 2, were tested in media containing 0.5 mg/L NAA or 0.5 mg/L IBA in the light After the 45 days of culture, the roots were harvested and the fresh weight recorded. The data were analyzed using ANOVA, with the confidence level for difference between treatment means set at $p \leq 0.05$.

RESULTS AND DISCUSSION

Root initiation and proliferation

The effects of different *in vitro* culture factors on root initiation and proliferation were studied. Root cultures of *P. radula* could be induced from all three explants (stem, petiole and leaf) on MS medium supplemented with NAA and IBA. Explants grown on media containing growth regulators achieved varying rates of root initiation success depending on the explant selected. Explants from stem segments performed better in root initiation and in producing roots compared to those derived from petiole or leaf (Table 1). The results obtained with *P. radula* differed from those reported in other species where, for example, petiole segments were reported to be the best tissue for the induction of hairy roots in *Centella asiatica* (Kim *et al.*, 2007) and in *Saussurea medusa* (Zhao *et al*., 2004). Mature leaves were the recommended initiating tissue for root culture of the chicory plant (Nandagopal and Ranjitha Kumari, 2007).

With *Rauwolfia serpentina* L., similarly, leaf explants produced roots on MS medium supplemented with growth regulators (Pandey *et al*, 2010). In the present study, well established roots were produced from stem explants supplied with the growth regulators tested singly or in combinations after 45 days on solid MS medium (Table 1). Of the various auxins supplemented, the response of the stem segments to the root initiation was better at concentrations below 1 mg/L of NAA or IBA. Treatment with 0.5 mg/L NAA, or 0.5 mg/L IBA treatments was best suited for root growth promotion, achieving root initiation of 97.8% and 91.0% respectively. Unlike Sivanesan and Jeong (2009) who found that the combination 1 g/L IBA and 0.5 g/L NAA induced the highest number of roots in *Plumbago zeylanicai*, none of the treatments that incorporated a combination of auxins (NAA + IBA or NAA + IAA) performed better than NAA alone in the present study (Table 1). A similar general trend in responses was observed for the average number of roots that developed per stem explant, with 0.5 mg/L being optimal for all three single auxins, NAA (8.1 roots), IBA (6.2 roots) or IAA (1.07 roots). These results reflect the observations of Jenifer *et al*. (2012) who noted that rooting of *Boerhaavia diffusa* was influenced by the particular type of auxin supplemented, and that NAA was more effective than IBA in this respect. The results from the present study are also consistent with those of Parvin *et al*. (2009), who found that 0.2 mg/L NAA induced maximum rooting, followed by 0.l mg/L of this auxin. Prasad *et al.* (2001) also reported that NAA influenced the number of roots induced in orchid. As noted in the results presented here, excessive auxins, including NAA, are detrimental to rooting. Similarly, it has been reported that a high concentration of NAA promoted the development of callus-like, short adventitious roots in *Karwinskia humboldtiana* (Kollarova *et al.*, 2004). Based on observations from the present study and from experimental results in the literature, therefore, solid MS medium with a lower concentration of NAA (0.5 g/L) was used for initiation of root culture of Pelargonium radula in the subsequent experiments.

Further investigations into root initiation were carried out using MS media of different concentrations, with the cultures maintained in darkness or light. Stem segments incubated on solid MS medium supplemented with 0.5 mg/L NAA showed more rapid induction of roots in darkness than in light (Figures 1c and 1d) for all MS strengths tested (Figure 2). The stimulatory effect of darkness and inhibitory effect of light condition on root induction have been well documented in other plant species (Sudha and Seeni, 2001; Seo *et al*., 2003). These results contrast with those of Hong *et al*. (2010) who favour continuous light for root induction of *Hyoscyamus niger* and total darkness for root proliferation. Similarly, Sivanesan and Jeong (2009 stated that when the cultures of *Plumbago zeylanica* were maintained under light conditions, the growth rate was less than those under darkness.

Table 1. Effect of different concentration of auxins on average root initiation incidence and average number of roots from different explants of *Pelargonium radula* cultured on solid MS medium

Plant growth regulator (mg/L)		Stem		Petiole		Leaf	
		Root initiation (%)	Number of roots per explant	Root initiation (%)	Number of roots per explant	Root initiation (%)	Number of roots per explant
IBA	0.2	81.67^{a}	4.10^{ed}	49.33^{f}	3.07^{efg}	9.00^{jk}	0.20^{i}
	0.5	91.00^{a}	6.20^{b}	29.67^{h}	4.30^{d}	10.33^{jk}	0.90^{i}
	1.0	60.00^{e}	6.20^{b}	0.00^{l}	0.07^{i}	5.00^{kl}	0.03^{i}
	3.0	49.00^{f}	2.20^{gh}	0.00^{l}	0.00^{i}	0.00^{l}	0.00^{i}
IAA	0.2	31.67^{h}	1.10^{hi}	0.33^{l}	0.00^{i}	0.00^{l}	0.00^{i}
	0.5	35.00^{h}	1.07^{hi}	10.00^{jk}	2.67^{fg}	0.00^{l}	0.00^{i}
	1.0	0.00^{l}	0.00^{i}	0.33^{i}	0.00^{i}	0.00^{l}	0.00^{i}
	3.0	0.00^{l}	0.00^{i}	0.00^{l}	0.00^{i}	0.00^{l}	0.00^{i}
NAA	0.2	85.00^{bc}	4.23^{be}	59.33^{e}	4.83^{cd}	12.00^{jk}	0.63^{i}
	0.5	97.67^{a}	8.10^{a}	61.00^{e}	3.83^{def}	15.33^{ij}	0.43^{i}
	1.0	$84.67b^{c}$	6.30^{b}	50.33^{f}	2.83^{fg}	20.67^{i}	0.57^{i}
	3.0	69.33^{d}	5.47^{bc}	42.0^{g}	4.13^{de}	9.67^{jk}	0.73^{i}
NAA : IBA	0.5 : 0.5	84.67^{a}	4.17^{b}	65.00^{bc}	2.17^{d}	0.33^{f}	0.03^{e}
	1.0 : 1.0	70.67^{a}	5.27^{a}	70.667^{b}	2.00^{d}	8.00^{e}	0.13^{e}
NAA : IAA	0.5 : 0.5	55.00^{d}	4.03^{b}	57.00^{d}	1.93^{d}	0.00^{f}	0.00^{e}
	1.0 : 1.0	65.00^{bc}	3.30^{bc}	58.00^{d}	2.40^{cd}	3.97^{ef}	$0.3e^{e}$

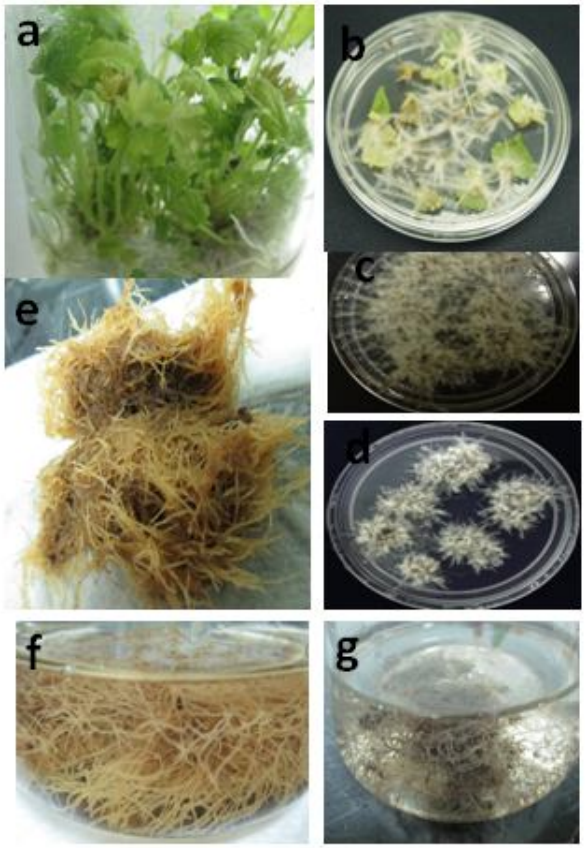

Figure 1. Induction and proliferation of adventitious roots of Pelargonium **radula. In vitro source material (a), initial stage of root initiation (b), development of root on solid medium with 15 g/L sucrose and incubated under light (c) or darkness (d).** Root **cultures harvested after 45 days in liquid culture with 0.5 mg/L NAA (e), Root cultures in liquid medium containing 0.5 mg/L NAA(f) and 0.5 mg/L IBA (g).**

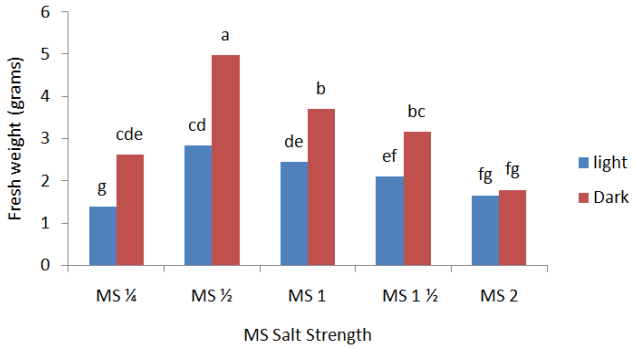

Figure 2. Effect of different MS salt strength on fresh weight of Pelargonium radula root culture that incubated for 45 days in dark and light conditions

In the present study, root initiation on solid half-strength MS medium in darkness produced the highest weight of roots (5.0 g), followed by full-strength MS (3.7 g) and 1½ strength MS (3.1 g) after 45 days of culture. Ameena *et al*. (2009) also observed that half strength MS was superior to the full strength growth medium in increasing root length in the presence of NAA, although IBA as a supplement performed better with full strength MS. Nevertheless, compatibility to the strength of culture media tends to vary with the plant species cultured. Thus, Sayeed and Shyamal (2005) found that 90% rooting was induced when *Gloriosa superba* explants were cultured on root induction medium consisting of half-strength MS salt supplemented with 1 mg/L IBA and 0.5 mg/L IAA. On the other hand, Jenifer *et al*. (2012) reported that roots in full strength MS gave the maximum fresh weight per culture of *Boerhaavia diffusa*. According to Fotopoulos and Sotiropoulos (2005), mineral concentration of the culture medium affects rooting characteristics and, as mentioned above, some researchers have reduced the strength of the culture medium used for rooting. The concentration of sucrose in the culture medium has also an important bearing on root initiation and growth. The addition of 15 g /L sucrose in the medium produced maximum root biomass (fresh weight 7.56 g) of *P. radula* cultured in darkness. However, further increase in sucrose concentration (30-75 g/L) adversely affected root biomass production (3.80-1.26g) (Figure 3). At sucrose concentrations of 60 - 75 g/L, roots were observed to be short and retarded. Our results are consistent with those of Hong *et al*. (2010), who reported that 60 g/L and 90 g/L sucrose reduced the root biomass production in *Hyoscyamus niger*. In addition, a decreasing trend in rooting was observed in *Orthosiphon stamineus* cultures when the concentration of sucrose was increased from 30-70 g/L (Anna *et al*, 2009).

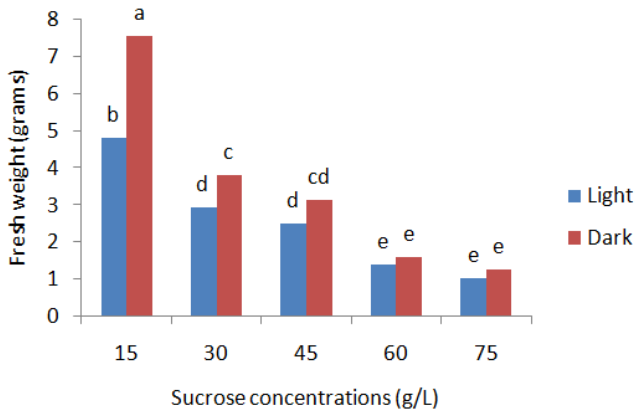

Figure 3. Effect of different sucrose concentrations on fresh weight of Pelargonium radula roots in cultures after 45 days in light or darkness

According to Borisjuk *et al*. (1998), the presence of sucrose in the induction phase may cause more cells to be involved in root induction, thus improving the rooting response. Findings by Jung *et al*. (2005) that an increase in sucrose level from 10 to 30 g/L markedly enhanced root growth in *Panax ginseng* support this proposition. Nevertheless, further increase in sucrose concentration repressed *Panax* root development. Kevers *et al*. (1999) similarly reported 30 g /L sucrose to be optimal for root biomass production in *Panax ginseng* and *Panax quinquefolium*. Similarly, Cheng *et al*. (1992) stated that sucrose concentrations at the range of 20-30 g/L were beneficial for rooting of *Eucalyptus sideroxylon*. On the other hand, Duong *et al*. (2001) found that 30-40 g/L of sucrose was already too high and tended to limit rooting of *Lilium longiflorum* in stem culture. Sucrose generally exerts osmotic pressure while, as a carbon source, it influences productivity of the plant. However, the carbon requirement varies according to plant species (Liu and Cheng, 2008). According to Anna *et al*. (2009), decrease in rooting efficiency with sucrose concentration higher than 30 g/L could be due to excessive amount of sucrose dissolved in the culture medium, giving rise to higher osmotic potential in culture medium than in the explants, which resulted in water stress.

Optimization and establishment of root cultures

Supplementations of 0.5 or 1.0 mg/L NAA, IAA and IBA were tested for their effect on adventitious root proliferation in MS liquid medium containing 3% sucrose. Among the tested auxins, 0.5 mg/L NAA was found to be the most potent in triggering adventitious root initiation and growth from the P. radula explants (Figure 1e, f). About 13 g of roots were obtained in cultures maintained either in light or in darkness (Table 2). Auxins tested in this study also affected the root morphology that is important in implementing mass production of root cultures. Treatment with IBA (Figure 1g) resulted in thick and

Table 2. Effect of NAA and IBA supplementation on root development of *Pelargonium radula* cultured in liquid medium

Plant growth Regulator (mg/L)		Fresh weight of root culture (grams)	
NAA	IBA	Light	Dark
0	-		
0.2	-	5.25^{de}	6.16^{cd}
0.5	-	13.42^{a}	13.13^{a}
1.0	-	7.17^{c}	9.32^{b}
1.5	-	2.91^{fg}	5.25^{de}
-	0.2	2.17^{gh}	3.06^{fg}
-	0.5	4.21^{ef}	3.64^{efg}
-	1.0	2.81^{fg}	1.87^{gh}
-	1.5	1.95^{gh}	0.76^{h}

Values are fresh weight of roots (g) after incubation for 45 days in light or darkness

long roots but the number of newly branched lateral roots was very few. In contrast, roots induced by NAA supplementation were thick and short in *Pueraria lobata* (Theim, 2003). The results in this study are in agreement with those obtained by Hasancebi *et al.* (2010) where maximum root fresh weight of *Astragalus chrysochlorus* was observed in liquid medium supplemented with 0.5 mg/L NAA. Similar results were also observed in *Robinia psuedoacacia* where NAA was found to be the most effective auxin to induce adventitious rooting (Swamy, 2001). Root proliferation in liquid medium was higher at lower concentrations of NAA, and gradually decreased when NAA concentration was increased. In the present study, root proliferation was strongly suppressed at higher concentrations of NAA and IBA (above 0.5 mg/L), an observation that has similarly been reported by Choffe *et al*. (2000). In contrast, Hossain *et al*. (2003) stated that among the three auxins (NAA, IBA and IAA) tested for root induction of *Zyziphus jujuba*, 1.0 mg/L IBA was found to be more effective in root production compared to others while inclusion of NAA (0.5-2.0 mg/L) in the medium resulted in a low rate of rooting. Different strengths of MS in liquid medium were compared for adventitious root proliferation. Adventitious roots in full-strength MS liquid medium with supplementation of 0.5 mg/L NAA was most effective in enhancing rooting, whereas other concentrations of the MS medium did not perform as well (Figure 4).

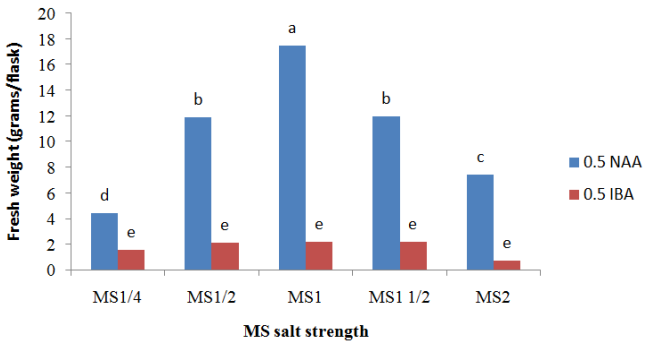

Figure 4. Effect of different MS concentration of liquid media supplemented with NAA or IBA on fresh weight of Pelargonium radula roots in cultures incubated for 45 days under light

Substituting NAA with IBA was generally ineffective. These results are in agreement with the findings of Jenifer *et al.* (2012), who demonstrated that full-strength MS liquid with NAA enhanced adventitious roots production in *Boerhaavia diffusa*, whereas treatment with half strength MS showed retardation in root growth. According to Polisetty *et al.* (1996), half and quarter strength MS media are better for *in vitro* rooting of chickpea than full-strength MS salts that function better in liquid media than in solid media (Fernandez *et al*., 1998). Many studies point to NAA activating cell division and enhancing adventitious root induction and elongation, for example in *Lycopersicon esculentum* (Taylor *et al.*, 1998). Pandey *et al*. (2010), observed good root induction in *Rauwolfia serpentine* when NAA was supplemented, but this advantage was compromised when a cytokinin (BAP) was also added. Notwithstanding the fact that auxins tend to inhibit root elongation, Pandey *et al*. (2010) assert that the production of lateral roots is important for rapid growth, and is responsible for higher biomass in the plant system. Reduced root growth might be due to the accumulation of endogenous auxins with each subculture.

Conclusion

The overall results show that half strength solid MS media supplemented with 0.5 mg/L NAA and 15 g/L sucrose effectively induces rooting from stem explants of *P.radula* cultured in total darkness. On the other hand, liquid full strength MS medium is most effective in promoting root proliferation. These experimental parameters will be referred to in future studies on secondary metabolite production in root cultures of *P. radula*.

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