

Silver nitrate induced rooting and flowering *in vitro* on rare rheophytic woody medicinal plant, *Rotula aquatica* Lour.

M Chithra¹, K P Martin^{2*}, C Sunandakumari¹ and P V Madhusoodanan¹

¹Department of Botany and ²Department of Biotechnology, University of Calicut, Calicut 673 635, India

Received 6 May 2003; accepted 8 August 2003

Silver nitrate induced enhanced rooting and flowering *in vitro* was achieved on *Rotula aquatica* L., a rare rheophytic woody aromatic medicinal plant. Solid and liquid nature of the medium, and growth regulators significantly influenced *in vitro* rooting and flowering. Half-strength MS liquid medium fortified with 2.69 μM α -naphthaleneacetic acid (NAA) was effective among different auxins, which induced a mean of 7.3 roots per shoot. Plantlets acclimatized after auxin alone induced rooting exhibited 80% survival in field conditions. Nevertheless, the established plantlets exhibited 50% loss within 3 months. Addition of 11.7 μM silver nitrate to half-strength MS liquid medium containing 2.69 μM NAA increased the number of roots to 16.8 per shoot. Plantlets established after rooting in silver nitrate supplemented medium facilitated 95% survival, and the established plantlets did not exhibit loss even after 3 months. Dipping of the basal end of shoots developed *in vitro* in NAA (2.69 μM) and silver nitrate (11.7 μM) solution (made in water) for 25 days induced a mean of 12.4 roots per shoot. These plantlets transferred to small pots facilitated 90% survival. Half-strength MS liquid medium containing 2.69 μM NAA and 11.7 μM silver nitrate induced flowering earlier. The flowers were morphologically and structurally similar to that on plants growing in field conditions.

Keywords: *Rotula aquatica*, liquid medium, plantlet survival, solid medium

IPC Code: Int. Cl.⁷ A 01 H 4/00, 5/02, 5/06

Introduction

Rotula aquatica Lour. belonging to the family *Boeraginaceae*, is a rheophytic woody aromatic medicinal shrub distributed in India, Sri Lanka, tropical South-East Asia and Latin America. The plant, known for its antilithic activity is rare, and usually grows in sandy and rocky beds of streams. Root tuber is the officinal part, and is a raw material source for many important Ayurvedic drug preparations. It is astringent, bitter, diuretic and also useful in cough, cardiac and blood disorders, dysuria, poison, fever, ulcers and uterine diseases¹. In Philippines, the decoction of root is used as a diuretic and laxative in piles, stones in bladder and in venereal diseases, and as sudorific. The root tubers contain the sterol, rhabdiol, and ureide allantoin².

The success and cost of micropropagation mainly relies on the per cent of plantlet survival in field conditions, which in turn depends on the efficient rooting of micropropagated shoots. Induction of rooting is an important step in micropropagation and genetic trans-

formation protocols, however, it has been often proved difficult, particularly in the case of woody plants^{3,4}. Adventitious root induction of woody species usually involves the treatments with exogenous auxins. Nevertheless, other factors including basal salt composition, explant physiology, light intensity, media condition (solid or liquid), silver nitrate, phloroglucinol have also been shown to affect adventitious rooting^{3,5,6}. Micropropagation and *in vitro* rooting using auxins and *ex vitro* rooting has been reported earlier on this species^{7,8}. However, plantlets established in field conditions in our previous study exhibited severe loss within three months after establishment due to less number of roots. Influence of growth regulators in the induction of *in vitro* flowering has also been reported^{4,9}. Thus, in the present study the authors describe better survival of *R. aquatica* through enhanced rooting using silver nitrate, and also the influence of silver nitrate in *in vitro* flowering.

Materials and Methods

Multiple shoots obtained through axillary bud multiplication on MS medium with 4.44 μM benzyladenine (BA) and 2.46 μM IBA⁸ have been used in

*Author for correspondence:

Tel: 0494-2401401144 Extn. 407; Fax: 0494-2400269

E-mail: martin@sify.com

the present study. The nutrient medium used for *in vitro* rooting and flowering studies consisted of the salts and vitamins of MS (1962) medium¹⁰. The medium was used either at full or half-strength as described. Both solid and liquid cultures were used. In liquid culture, the basal end of the healthy shoots having more than 4 cm height were kept in 10 ml of medium without support. Solid medium was gelled with 0.7% (w/v) agar. The medium was supplemented with various levels of different growth regulators viz. α -naphthaleneacetic acid (NAA)/indole-3-acetic acid (IAA)/indole-3-butyric acid (IBA). Different levels of silver nitrate (5.89-17.7) were also added to half-strength MS medium either singly or in combination with NAA. All the media were prepared with 3% (w/v) sucrose and their pH was adjusted to 5.8 before adding the agar. They were autoclaved at a pressure of 1.06 kg cm⁻². The cultures were incubated at 25±2°C with 16/8 hrs photoperiod under white fluorescent tubes (25 μ mol m⁻² s⁻¹).

Rooting was also attempted outside the laboratory by keeping the basal cut end of healthy shoots (height above 4 cm) from the shoot multiplication medium dipped in 2.69 μ M NAA and 11.7 μ M silver nitrate (this combination exhibited best *in vitro* rooting) containing solution (made in tap water) for 25 days. Dipping was given by keeping five shoots in test tubes containing 10 ml of 2.69 μ M NAA and 11.7 μ M silver nitrate solution. The test tubes containing the shoots with dipped basal ends were kept open at room temperature (32±2°C).

The shoots rooted in medium containing NAA alone and in combination with silver nitrate, and also in *ex vitro* dipped method were separately transferred to small pots containing soil and sand (1:1). The transplanted rooted shoots were kept at room temperature. The *in vitro* derived plantlets were initially covered with moistened polyethylene bags. The per cent survival of plantlets established in field conditions in each case was recorded independently after 3 months. All the experiments were set up in a completely randomised design. Twenty replicates were raised for each treatment, and all experiments were repeated once (total of 40 replicates). Mean values were compared using Duncan's multiplication range test.

Results and Discussion

Healthy shoots developed on MS medium with 4.44 μ M BA and 2.46 μ M IBA, having more than 4 cm height, transferred on MS basal solid medium induced mean of 2.9 roots per shoot, but only on 35% shoots. Half-strength MS medium supplemented with lower levels of auxins (NAA/IAA/IBA) alone favoured better rhizogenesis (Table 1). Liquid medium was superior to solid medium for the induction of rooting. Half-strength MS solid medium enriched with NAA at 2.69 μ M induced roots at the best, which developed a mean of 6.1 roots/shoot (Table 1). The roots were with numerous root hairs. Increase of NAA induced callus from the base of the shoot, but later developed more than 6 short (< 1 cm) roots. In the induction of roots, NAA was followed by IBA, and IAA (Table 1). The induction efficacy of roots on

Table 1—*In vitro* rooting of *R. aquatica* using half-strength MS medium supplemented with different auxins and silver nitrate alone or in combination

Growth regulators (μ M)			Silver nitrate (μ M)	Percentage of rooting		Number of roots per shoot	
IBA	NAA	IAA		Solid	Liquid	Solid	Liquid
	Basal medium			40	45	3.2 ^{fg}	3.9 ^h
0.49				75	80	3.7 ^f	4.1 ^g
2.46				95	95	4.3 ^e	5.3 ^f
4.90				90	90	2.1 ^h	2.9 ⁱ
	0.54			75	80	4.4 ^e	6.1 ^e
	2.69			90	95	6.1 ^c	7.3 ^c
	5.37			85	90	6.0 ^c	7.1 ^c
		0.57		80	80	3.6 ^f	4.1 ^g
		2.85		95	95	4.2 ^e	5.2 ^f
		5.71		80	85	2.1 ^h	2.7 ⁱ
			5.89	75	75	3.5 ^f	4.2 ^g
			11.7	80	80	4.3 ^e	5.3 ^f
			17.7	85	85	3.7 ^f	4.3 ^g
	2.69		5.89	85	85	7.9 ^b	12.1 ^b
	2.69		11.7	95	95	10.3 ^a	16.4 ^a
	2.69		17.7	85	90	5.4 ^{cd}	7.3 ^c

Mean of 20 replicates. Means followed by the same letters are not significantly different at 5% level. Growth period 40 days.

solid medium with NAA was similar to the previous study⁸. *In vitro* rooting using liquid culture supplemented with NAA (2.69 μ M) was superior to solid culture, and induced a mean of 7.3 roots per shoot.

Half-strength MS medium supplemented with various concentrations of silver nitrate alone also induced roots, but the number of roots was less than that with the optimal level of NAA (Table 1). Addition of silver nitrate at different levels to half-strength MS medium containing optimal level of NAA (2.69 μ M) significantly augmented the induction of roots. Silver nitrate and NAA supplemented medium initiated roots within 10 days, while medium with auxin alone started to initiate roots after 14 days. Half-strength MS liquid medium with 2.69 μ M NAA and 11.77 μ M silver nitrate induced the highest number of roots, a mean of 16.4 roots (Table 1) with more than 5 cm length within in 21 days (Fig. 1A). The roots were healthier and much branched with numerous root hairs. Improved root induction by the synergy of an auxin and silver nitrate has been emphasized in apple¹¹, *Decalepis hamiltonii*⁵ and *Vanilla planifolia*⁶.

The use of liquid medium in the present study induced the highest number of roots, which were much longer and branched than that on solid medium of the same composition. In addition, the liquid medium facilitated faster and better growth of shoots. Improved induction of roots and overall growth of roots and shoots using liquid culture has been reported in *Holostemma ada-kodien*¹².

Basal cut end of shoots dipped in 2.69 μ M NAA and 11.7 μ M silver nitrate containing solution (made in water) at room temperature induced roots from 90% shoots. The shoots induced a mean of 12.4 roots per shoot within 25 days. The induced roots exhibited similar nature of that induced in liquid cultures *in vitro*. Rooting by this method reduced cost, and the acclimatization period compared to *in vitro* rooting methods.

The shoots on half-strength MS medium without silver nitrate never induced flowers *in vitro*. According to Handro¹³, low concentration of auxins is beneficial for *in vitro* flower induction. In contrast, medium with low concentration of auxins alone did not induce flowers in the present study. On medium with silver nitrate (5.87-17.7 μ M) alone, flowers were induced after 45 days. Shoots transferred to half-strength MS liquid medium supplemented with 2.69 μ M NAA and 11.7 μ M silver nitrate abreast to the induction of roots, flowered between 21 and 32 days,

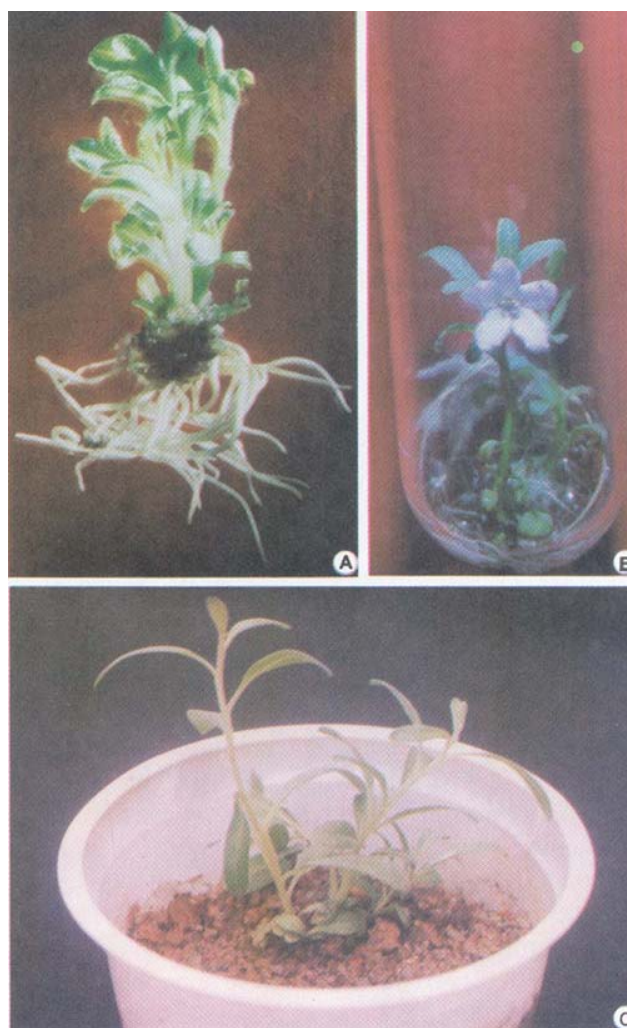


Fig. 1—*In vitro* rooting and flowering of *R. aquatica*; A, Roots developed from shoots dipped in half-strength MS liquid medium containing 2.69 μ M NAA and 11.7 μ M silver nitrate; B, Shoots with roots and flower in medium as above; and C, Plantlets established in small pot (45 days).

and mostly the flowers were in 2 or 3 terminating short lateral branches (Fig. 1B). On solid medium, flowering was delayed, and occurred after 35 days. Influence of silver nitrate in *in vitro* flowering has been reported in *Cichorium intybus*⁴. However, in the present study, synergy of auxin (NAA) and silver nitrate was superior for the induction flowers *in vitro*. Morphological and structural characters of the flowers were similar to that on field growing plants. The flowers were pink with five sepals, petals (with imbricate aestivation), and stamens.

Healthy plantlets obtained through different ways:

from NAA alone, NAA and silver nitrate containing medium, and *ex vitro* dipped rooting method, were separately transferred to small pots containing soil and sand (1:1). The transferred plantlets revived growth within 12 days, grew well (Fig. 1C), and were subsequently transferred to field. Survival of plantlets in field conditions depended on the rooting medium, i.e. medium with NAA alone, NAA and silver nitrate, and *ex vitro* rooting. Shoots rooted on medium having NAA alone facilitated 80% establishment. This was in concurrence with the earlier reports on *R. aquatica*^{7,8}. Nevertheless, 50% loss of field established plantlets was observed within three months, and was due to less number of roots, and inability to produce new roots. Plantlets obtained from auxin and silver nitrate containing medium *in vitro*, and also from NAA and silver nitrate solution made in water exhibited 95 and 90% survival, respectively; and all of them survived and grew well even after three months. Greater survival of the shoots rooted using auxin and silver nitrate may be due to the increased number of roots, and the persistence of root stimulant to induce new roots after establishment. Morphological characters of the plantlets such as stem colour, leaf morphology, growth pattern, flower colour, etc. established in field conditions were similar to that of the source plant.

In the present study, enhanced rooting, and better survival of rare rheophytic woody aromatic medicinal plant, *R. aquatica* has been achieved.

References

- 1 Sivarajan V V & Balachandran I, *Ayurvedic drugs and their plant sources* (Oxford & IBH Publishing House, New Delhi) 1994, 358-359.
- 2 Anonymous, *Wealth of India, Raw materials*. Vol. IX (Publications & Information Directorate, CSIR, New Delhi) 1972, 80.
- 3 Ainsley P J, Collins G G & Sedgley M, *In vitro* rooting of almond (*Prunus dulcis* Mill.), *In Vitro Cell Dev Biol Plant*, 37 (2001) 778-785.
- 4 Bais H P, Sudha G & Ravishankar G A, Influence of putrescine, silver nitrate and polyamine inhibitors on the morphogenetic response in untransformed and transformed tissue of *Cichorium intybus* and their regenerants, *Plant Cell Rep*, 20 (2001) 547-555.
- 5 Reddy B O, Giridhar P & Ravishankar G A, *In vitro* rooting of *Decalepis hamiltonii* Wight. & Arn., an endangered shrub by auxins and root prompting agents, *Curr Sci*, 81 (2001) 1479-1482.
- 6 Giridhar P, Reddy B O & Ravishankar G A, Silver nitrate influences *in vitro* shoot multiplication and root formation in *Vanilla planifolia* Andr., *Curr Sci*, 81 (2001) 1166-1170.
- 7 Sebastian D P, Benjamin S & Hariharan M, Micropropagation of *Rotula aquatica* Lour.—An important woody medicinal plant, *Phytomorphology*, 52 (2002) 137-144.
- 8 Martin K P, Rapid *in vitro* multiplication and *ex vitro* rooting of *Rotula aquatica* Lour., a rare rheophytic woody medicinal plant, *Plant Cell Rep*, 21 (2003) 415-420.
- 9 Lin C-C, Lin C-S & Chang W-C, *In vitro* flowering of *Bambusa edulis* and subsequent plantlet survival, *Plant Cell Tissue Organ Cult*, 72 (2003) 71-78.
- 10 Murashige T & Skoog F, A revised medium for rapid growth and bioassays for tobacco tissue cultures, *Physiol Plant*, 15 (1962) 473-497.
- 11 Ma J H, Yao J L, Cohen D & Morris B, Ethylene enhance *in vitro* root formation from apple shoot culture, *Plant Cell Rep*, 17 (1998) 211-214.
- 12 Martin K P, Rapid propagation of *Holostemma ada-kodien* Schult., a rare medicinal plant, through axillary bud multiplication and indirect organogenesis, *Plant Cell Rep*, 21 (2002) 112-117.
- 13 Handro W, Effect of some growth regulators on *in vitro* flowering of *Streptocarpus nobilis*, *Plant Cell Rep*, 2 (1983) 133-136.