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In Vitro Flowering in *Rauvolfia serpentina* (L.) Benth. ex. Kurz.

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ABSTRACT

Rauvolfia serpentina Benth. is an economically important medicinal plant renowned for curing cardiovascular diseases and hypertension, its pharmacological activity being due to the presence of different alkaloids. Multiple shoot regeneration and flower induction *in vitro* have been achieved in this study using combinations of cytokinin and auxin. Flowering was induced for the first time ever in Murashige and Skoog medium supplemented with 2.22 μM benzyl adenine (BA) + 2.32 μM kinetin (Kin) + 0.54 μM α-naphthalene acetic acid and 2.22 μM BA + 4.65 μM Kin under a 12-hr photoperiod.

Keywords: micropropagation, multiple shoot regeneration, Murashige and Skoog medium, plant growth regulators
Abbreviations: BA, 6-benzyl adenine; **GA_3**, gibberelic acid; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **Kin**, kinetin; **MS**, Murashige and Skoog; **NAA**, α-naphthalene acetic acid; **PGR**, plant growth regulator

INTRODUCTION

Five species of the genus *Rauvolfia* (family Apocynaceae) have been reported from India (Bhattacharjee 1998). Among them, *R. serpentina* (L.) Benth. ex. Kurz. is the most extensively studied and highly exploited species; it possesses a total alkaloid content between 0.7 and 3.0% of total root dry mass (Dhiman 2006) and a reserpine content at 0.1% of dry root (Anonymous 1998; Anitha and Kumari 2007). Isolated reserpine is used in modern medicine to treat different cardiovascular diseases, hypertension and psychological disorders, and has been found to be more potent than the crude drug prepared from the roots (Pullaiah 2002). Due to overexploitation and lack of organised cultivation, wild populations have declined rapidly and the species is now listed as endangered (Swarup and Arora 2000). With an increasing worldwide demand for plant-derived medicine and formulations, there has been a concomitant increase in the demand for raw materials. Hence there is a need to develop an approach for ensuring the availability of raw materials with a consistent quality of drugs from regular and viable sources. Rapid mass propagation of *R. serpentina* through tissue culture has already been reported by many authors. Bhatt *et al.* (2008) optimised a protocol for rapid multiplication for *R. serpentina*, Goel *et al.* (2009) cultured *R. serpentina* in liquid Murashige and Skoog (MS; Murashige and Skoog 1962) medium and quantified the alkaloids produced. Roja and Heble (1996) also isolated indole alkaloids from rapidly growing *R. serpentina* cultures. The literature reveals that *in vitro* flowering of *Rauvolfia* species is scanty. Sharma *et al.* (1999) observed *in vitro* flowering of *R. tetraphylla* in shoot multiplication medium containing 6-benzyl adenine (BA) and kinetin (Kin) but the response was poor. Anitha and Kumari (2006) observed *in vitro* flowering in MS medium containing BA and gibberellic acid (GA_3). However, *in vitro* flowering in *R. serpentina* has not yet been reported in the literature. This study was undertaken with an objective to standardise a protocol for *in vitro* mass propagation with special emphasis on *in vitro* flowering.

MATERIALS AND METHODS

Extran, sucrose and agar were purchased from E. Merck (India) Ltd., Mumbai, India. $HgCl_2$ was purchased from SISCO Research Laboratories Pvt. Ltd., Mumbai, India. Ethanol was purchased from Bengal Chemicals and Pharmaceuticals Ltd., Kolkata, India. Plant growth regulators (BA, Kin, α-naphthalene acetic acid (NAA)) were purchased from Merck Specialities Pvt. Ltd., Mumbai, India.

Apical buds 1–2 cm long and nodal bud explants were collected from 2–3-years-old field-grown plants of the Medicinal Plants Garden of the Department of Botany, Kalyani University, Kalyani, Nadia, West Bengal. The surface of explants was cleaned with 5% Extran for 10 min then washed thoroughly with running tap water. Rinsed explants were surface sterilised with 0.2% $HgCl_2$ (w/v) for 6-7 min in a laminar airflow chamber and washed twice with sterile distilled water. Explants were then dipped in 70% ethanol for 1 min and finally washed thoroughly with sterile distilled water 4-5 times to remove all residual traces of sterilants. The basal nutrient medium consisted of MS salts (full macro- and micronutrients) and vitamins and 3% (w/v) sucrose and was gelled with 0.8% (w/w) agar. For regeneration, BA and Kin were used in several permutations indicated in **Table 1**, which were altered during secondary culture (**Table 2**) that included the auxin NAA (**Tables 1, 2**). The pH of the media was adjusted to 5.7 and the medium was sterilised at 2.1756×10^{-3} Pa for 15 min. All cultures were placed at 24 ± 2°C in a 12-hr photoperiod with light intensity of 40 μM m^{-2} sec^{-1} under cool white fluorescent tubes (Model LIFEMAX-A 73, Philips).

Results were recorded periodically and the data were subjected to statistical analysis. For each treatment 10 replicates were used and the mean values derived from the experiments were subjected to one-way analysis of variance (ANOVA). Means were separated by Duncan's multiple range test (DMRT) using SPSS software (Statistical Package for the Social Sciences) version 10.0 (LEAD Technologies Inc., Chicago, USA) at $P = 0.05$.

RESULTS

Bud break was observed 10 days after inoculation from both bud explants. The maximum number of shoot buds

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Short Communication

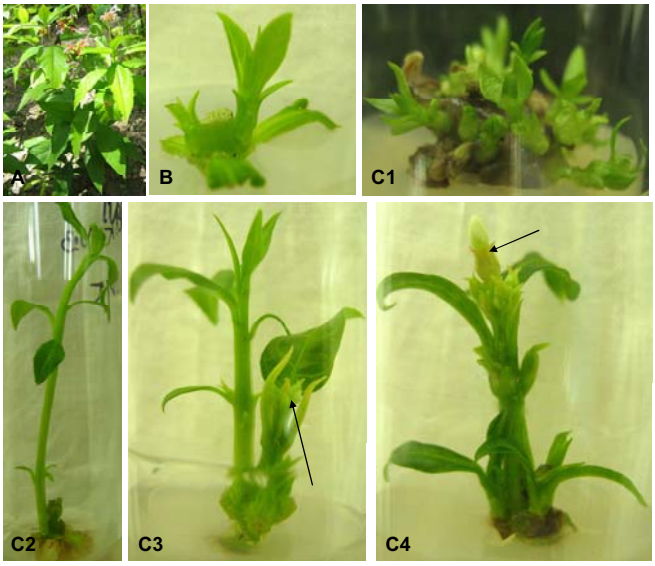

Fig. 1 ***Rauvolfia serpentina*** **L.** ***in vitro*** **flowering.** (**A**) Whole plant. (**B**) Primary culture. (**C**) Secondary culture: 10-days-old culture (C1), 30 days old culture (C2), flower bud induction (C3) and flowering plantlet (C4). Arrow indicates *in vitro* flower bud.

Table 1 Primary culture of *R. serpentina*.

Growth regulators (µM)			Growth parameters	
BA	Kin	NAA	No. of shoots	Shoot length (cm)
2.22	2.32	-	1.2 ± 0.13 e	3.4 ± 0.16 cd
2.22	4.64	-	3.6 ± 0.27 a	4.1 ± 0.18 b
2.22	9.29	-	2.1 ± 0.18 d	4.1 ± 0.18 b
4.44	2.32	-	1.1 ± 0.13 e	3.5 ± 0.17 cd
4.44	4.64	-	3.1 ± 0.18 ab	3.1 ± 0.18 de
4.44	9.29	-	2.9 ± 0.13 bc	4.2 ± 0.13 b
8.88	2.32	-	2.6 ± 0.27 bcd	3.4 ± 0.16 cd
8.88	4.64	-	2.1 ± 0.18 d	3.8 ± 0.26 bc
8.88	9.29	-	1.1 ± 0.13 e	2.7 ± 0.21 e
2.22	4.64	0.54	3.4 ± 0.22 a	6.1 ± 0.18 a
2.22	4.64	1.61	3.1 ± 0.18 ab	4.2 ± 0.20 b
2.22	4.64	2.69	2.5 ± 0.17 cd	3.5 ± 0.17 cd

Total number of explants used for each concentration = 10 (bulked from both explant types); Each experiment was repeated in triplicate; Values are presented as mean ± standard error. Means within a column followed by the same letter are not significant at $P = 0.05$ according to DMRT (Duncan's multiple range test)

(3.6 ± 0.27) formed on MS medium supplemented with BA (2.22 µM) and Kin (4.65 µM) during regeneration (primary culture) (**Fig. 1B**, **Table 1**). In primary cultures, maximum shoot length 6.1 ± 0.18 cm was observed in regeneration medium supplemented with BA (2.22 µM), Kin (4.65 µM) and NAA (0.54 µM) (**Table 1**). Regenerated plantlets were transferred to maintenance medium (secondary culture) after 40 days. Among the different plant growth regulator (PGR) combinations tested during secondary culture (**Fig. 1C**, 1-4), three combinations gave satisfactory results: BA (2.22 µM) + Kin (2.32 µM), BA (2.22 µM) + Kin (4.65 µM) and BA (2.22 µM) + Kin (2.32 µM) + NAA (0.54 µM) (**Table 2**). Among these, the latter gave the best result, in which a maximum number of microshoots was observed (28.4 ± 0.22) (**Fig. 1C**, 2**, Table 2**). Maximum shoot length (**Fig. 1C**, 3) 5.3 ± 0.15 cm was achieved by the same PGR combination, i.e., BA (2.22 µM) + Kin (2.32 µM) + NAA (0.54 µM).

Flower buds were induced (**Fig. 1C**, 4) in two PGR combinations, BA (2.22 µM) + Kin (4.65 µM) and BA (2.22 µM) + Kin (2.32 µM) + NAA (0.54 µM) after an incubation period of 30 days after subculturing (**Tables 2, 3**). Flower bud induction was observed in 75 and 90% of plantlets in these two PGR combinations, respectively (**Table 3**). Interestingly, those plantlets which induced flowers produced at least 70% fewer microshoots than the other cultures with the same PGR medium composition (**Table 3**).

Table 2 Secondary culture of *R. serpentina*.

Plant growth regulators (µM)			Growth parameters	
BA	Kin	NAA	No. of microshoots	Shoot length (cm)
0.44	0.46	-	5.1 ± 0.18 j	2.6 ± 0.22 bcde
0.44	2.32	-	3.4 ± 0.16 k	2.1 ± 0.18 defghi
0.44	4.64	-	5.0 ± 0.15 j	1.8 ± 0.20 fgh
0.44	6.97	-	4.5 ± 0.17 j	2.3 ± 0.21 cdef
2.22	0.46	-	2.0 ± 0.15 l	1.5 ± 0.17 hi
2.22	2.32	-	25.1 ± 0.18 b	2.2 ± 0.20 cdefg
2.22	4.64	-	21.5 ± 0.22 c*	2.8 ± 0.25 bc
2.22	6.97	-	14.6 ± 0.22 d	2.6 ± 0.22 bcde
4.44	0.46	-	6.1 ± 0.18 i	2.2 ± 0.13 cdefg
4.44	2.32	-	4.8 ± 0.20 j	2.0 ± 0.21 efgh
4.44	4.64	-	8.6 ± 0.22 f	1.2 ± 0.13 i
4.44	6.97	-	10.2 ± 0.13 e	2.4 ± 0.16 cdef
6.66	0.46	-	5.0 ± 0.21 j	2.7 ± 0.26 bcd
6.66	2.32	-	3.7 ± 0.26 k	2.8 ± 0.25 bc
6.66	4.64	-	5.1 ± 0.18 j	1.6 ± 0.22 ghi
6.66	6.97	-	7.7 ± 0.21 g	2.6 ± 0.22 bcde
2.22	2.32	0.54	28.4 ± 0.22 a*	5.3 ± 0.15 a
2.22	2.32	1.61	15.1 ± 0.18 d	3.1 ± 0.18 b
2.22	2.32	2.69	7.1 ± 0.23 h	1.2 ± 0.13 i

* *In vitro* flowering

Total number of explants used for each concentration = 10 (bulked from both explant types); Each experiment was repeated in triplicate; Values are presented as mean ± standard error. Means within a column followed by the same letter are not significant at $P = 0.05$ according to DMRT (Duncan's multiple range test)

DISCUSSION

Microshoot development

Auxin, when combined with a cytokinin, plays a role in the elongation of regenerated shoots. According to Ilahi *et al.* (2007) and Bhatt *et al.* (2008), such an auxin + cytokinin combination produces better results in direct shoot regeneration and increasing shoot length in *Rauwolfia* then when used individually. In the present work, a maximum number of microshoots were observed with the BA + Kin combination in primary culture and with the BA + Kin + NAA combination in secondary culture. Ahmad *et al.* (2002), Kataria and Shekhawat (2005), Arif *et al.* (2008) and Goel *et al.* (2009) also observed maximum number of microshoots for *Rauwolfia* spp. in auxin + cytokinin combinations but they used only one cytokinin together with an auxin although Patil and Jayanthi (1997) used a single cytokinin, BA (8.88 µM) for multiple shoot induction. Ahmad (2002) produced multiple shoots with BA (11.1 µM) and NAA (0.54 µM) and observed shoot elongation with BA (8.88 µM) and NAA (0.27 µM). We thus concluded that NAA plays a role in shoot elongation. Kataria and Shekhawat (2005) produced multiple shoots from nodal explants by axillary bud proliferation with BAP (10 µM) and indole-3-acetic acid (IAA) (0.5 µM). Arif *et al.* (2008) observed better results for direct regeneration with indole-3-butyric acid (IBA) (0.62 µM) and BAP (4.44 µM). Goel *et al*. (2009) observed *in vitro* shoot multiplication with BAP (4.44 µM) and NAA (0.54 µM). However, the use of two cytokinins simultaneously is rare and was observed by Balaraju (2008) who induced highest number of multiple shoots in *Swertia chirata* Buch.-Ham. *ex* Wall using the combination of BAP (4.44 µM) and Kin (0.46 µM). Another example of the use of BAP and Kin simultaneously for high frequency multiple shoot development was observed in *Basilicum polystachyon* (L.) Moench (Amutha 2008). Verma and Singh (2007) produced a maximum number of shoots in both cotyledonary node and shoot apex explants of *Brassica campestris* L. var. *Bhavani* with BA (11.1 µM), IAA (5.71 µM) and Kn (2.32 µM).

Table 3 *In vitro* flowering response in *R. serpentina.*

Plant growth regulators (µM)			Growth parameters				
BA	**Kin**	**NAA**	**Flowering response (%)***	**No. of microshoots**	**Shoot length (cm)**	**No. of flower buds produced**	**No. of flowers that matured**
2.22	4.64	-	75	3.6 ± 0.24	2.4 ± 0.27	6.7 ± 0.21	1.6 ± 0.16
2.22	2.32	0.54	90	2.7 ± 0.26	2.9 ± 0.23	5.5 ± 0.22	1.1 ± 0.15

* mean % explants with a flowering response
All values bulked from both explant types

Flowering

In vitro flowering is a complex reaction in response to abiotic cues, primarily PGR combinations, explant type, photoperiod and light spectral quality (Stephen and Jayabalan 1998; Bernier and Perilleux 2005; reviewed in Sudhakaran *et al.* 2006). The incidence of flowering was probably induced by the exogenous supply of PGRs, which in turn might have raised the endogenous content to a level required for triggering flowering as was opined by Verma and Singh (2007) during *in vitro* flower bud induction in *Brassica campestris.* Maximum flowering (50%) was noted in the shoots from cotyledonary nodes exposed to IBA (7.38 µM), IAA (5.71 µM) and Kn (2.32 µM). Anitha and Kumari (2006) produced a maximum number of microshoots with only BAP (4.44 µM), although they induced flowering in *R. tetraphylla* with BAP and GA_3, which was in contrast with a previous study on *in vitro* flowering of the same species by Sharma *et al.* (1999), who reported optimum response for *in vitro* flowering with BAP and cytokinin combination, which is consistent with our report. In *Murraya paniculata* (L.) Jack, BAP alone induced floral bud formation (Jumin and Ahmed 1999). In many *in vitro* flowering experiments BAP has been used alone or in combination with other PGRs and it might play a major role in flower bud formation and maturation (Anitha and Kumari 2006). This is further supported by these studies: Sudhakaran and Sivasankari (2002) in *Ocimum basilicum* L., Wang *et al.* (2001) in *Momordia charantia* L., Mandal and Sheeja (2003) in *Lycopersicon esculentum* Mill., and Hee *et al.* (2009) in *Dendrobium* sp.

Although not tested in this study, the importance of photoperiod for *in vitro* flowering has been demonstrated in *Murraya paniculata* plantlets, derived from protoplasts, which only flowered in a 16-hr photoperiod but not in continuous darkness (Jumin and Nito 1995). The effects of photoperiod on vegetative and reproductive development were also demonstrated in *Psygmorchis pusilla* (Vaz *et al.* 2004). Zimmerman *et al.* (1985) were of the opinion that the interaction of carbohydrate and other nutritional factors with endogenous PGRs can influence some biological parameters that are altered when a plant changes from the juvenile to the mature phase.

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