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

Subhasish Mondal¹ • Jaime A. Teixeira da Silva² • Partha Deb Ghosh²*  

1. Cyto-genetics and Plant Breeding Section, Plant Tissue Culture and Biotechnology Research Unit, Department of Botany, University of Kalyani, Kalyani-741235, Nadia, West Bengal, India  
2. Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Ikenobe 2393, Kagawa-ken, 761-0795, Japan  

**Corresponding author:** *pdggene@rediffmail.com, subhasish1982@sify.com*

**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; GA3, 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 over-exploitation 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. Bhattacharjee 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 gibberelic acid (GA3). 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. HgCl2 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% HgCl2 (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/v) 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 medium was adjusted to 5.7 and the medium was sterilised at 2.1756×10⁻¹³ 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
Flower bud induction was observed in 75 and 90% of plantlets which induced flowers progressively with BA (2.22 μM) + Kin (2.32 μM) + 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), three combinations gave satisfactory results: BA (2.22 μM) + Kin (2.32 μM) + NAA (0.54 μM) (Fig. 1C, 2) and BA (2.22 μM) + Kin (4.65 μM) + NAA (0.54 μM) (Fig. 1C, 3). A maximum number of microshoots were observed with the BA + Kin combination, i.e., BA (2.22 μM) + Kin (2.32 μM) + NAA (0.54 μM). However, the use of two cytokinins simultaneously for high frequency multiple shoot induction was observed by Balaraju (2008) who 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-butyrice 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 Basileiculum 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. Bravani with BA (11.1 μM), IAA (5.71 μM) and Kin (2.32 μM).

**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 Rauvolfia than 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 Basileiculum 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. Bravani with BA (11.1 μM), IAA (5.71 μM) and Kin (2.32 μM).
Table 3 In vitro flowering response in R. serpentina.

<table>
<thead>
<tr>
<th>Plant growth regulators (μM)</th>
<th>BA</th>
<th>Kin</th>
<th>NAA</th>
<th>Flowering response (%)*</th>
<th>No. of microshoots</th>
<th>Shoot length (cm)</th>
<th>No. of flower buds produced</th>
<th>No. of flowers that matured</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.22</td>
<td>4.64</td>
<td>-</td>
<td>75</td>
<td>3.6 ± 0.24</td>
<td>2.4 ± 0.27</td>
<td>6.7 ± 0.21</td>
<td>1.6 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>2.22</td>
<td>3.22</td>
<td>0.54</td>
<td>90</td>
<td>2.7 ± 0.68</td>
<td>2.9 ± 0.23</td>
<td>5.5 ± 0.22</td>
<td>1.1 ± 0.15</td>
</tr>
</tbody>
</table>

* mean % Explants with a flowering response

Table 3 In vitro flowering response in R. serpentina.

Flowing

In vitro flowering is a complex reaction in abi-otropes, 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 GA3, 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 Momordica charantia L., Mandal and Sheeba (2003) in Lycopersicum esculentum Mill., and Hsu 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 Psychotria pallida (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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