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Micropropagation and total alkaloid extraction of Indian snake root (*Rauwolfia serpentina*)

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ABSTRACT

An efficient and reproducible protocol for clonal propagation was developed for Indian snake root (*Rauwolfia serpentina* L. Benth. ex. Kurtz.) through indirect and direct organogenesis using nodal segment, shoot apices and leaves as explants. For indirect organogenesis using leaf and nodal segments, most efficient callus induction was obtained on MS + 2.0 mg /litre 2, 4-D + 1.0 mg /litre BAP. Proliferated calli further showed highest regeneration response in MS + 1.0 mg / litre BAP + 0.1 mg /litre KIN + 0.1 mg /litre GA_3. However, for direct organogenesis using shoot apices and nodal segments, most efficient regeneration was observed in MS +1.0 mg /litre BAP + 0.1 mg /litre KIN + 0.1 mg /litre GA_3. For complete removal of contamination due to endophytic microflora, 6.0 g /litre bavistin and 100.0 mg /litre streptomycin was found most effective. Regenerated plantlets obtained both through direct and indirect organogenesis showed multiple shooting on MS + 1.0 mg /litre BAP + 0.1 mg /litre NAA and excellent rooting in MS + 1.0 mg /litre IBA + 1.0 mg /litre IAA. Well rooted plantlets were successfully acclimatized in greenhouse. Plantlets developed through shoot apices showed maximum height, number of shoots and number of leaves. Quantitative estimation of crude alkaloids from six weeks old regenerated plantlets was found similar to that obtained from leaves and stems of one year old field grown plant. The developed protocol can be used for *en masse* propagation and alkaloid extraction of *Rauwolfia serpentina*.

Key words: Alkaloids, Endangered species, Organogenesis, *Rauwolfia serpentina*, Shoot multiplication

Indian snake root (*Rauwolfia serpentina* L. Benth. ex Kurz.) is a small woody perennial medicinal herb. It is mainly distributed in Indian sub-continent, particularly in the sub-tropical regions of Himalaya (Dey and De 2010). *R. serpentina* contains some 50 monoterpene indole alkaloids (MIAs), of them reserpine, yohimbine, serpentine, deserpidine, ajmalicine, ajmaline, etc. are used to treat hypertension and breast cancer (Ebadi 2002, Baskarrajan *et al.* 2003). However, this plant is threatened to extinction due to its limited cultivation and over exploitation (Bhatt *et al.* 2008). Further, poor seed viability and low germination percentage (25–50%) because of the presence of cinnamic acid and its derivatives in the seeds, this plant is vegetatively propagated by root cutting which is not enough to replenish the current demand (Salma *et al.* 2008). With a remarkable potential for massive propagation and isolation of MIAs, *in vitro* culture is now in practice for various economically important plants (Joshi *et al.* 2009). High yield of secondary metabolites were also isolated with *in vitro* cultures (Anitha and Kumari 2006). In addition, multiple shoot culture is preferred method for the large-scale production of biomass and multiplication of existing stocks of germplasm (Joshi *et al.* 2009, Dey and De 2010).

Since late 20th century, *R. serpentina* became plant model system for investigating MIA production by *in vitro* culture techniques. However, field grown plants are still the main source of several MIAs (Pasquali *et al.* 2006). The chemical synthesis of these complex natural drugs is commercially not available, making field-grown plants the sole source of several bioactive alkaloids (Sudha *et al.* 2003). Although root is primary source of alkaloids in *R. serpentina*, previous studies

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reported around 21 different alkaloids in stem bark and 19 alkaloids in leaves (Ebadi 2002). Since now no work has been reported concerning total alkaloid production from leaves and stem of *in vitro* grown *R. serpentina* plants. Therefore, a detailed understanding of the alkaloid production at whole plant level offers important resources of commercially viable bioactive molecules *in vitro*. There is also an urgent need for mass propagation of *R. serpentina* to satisfy the increasing demand of important alkaloids and conservation of this endangered species. Keeping the above facts in mind, the present study was undertaken to investigate the *in vitro* propagation, including effect of exogenous plant hormones on direct and indirect organogenesis. We also examined the potential of total crude alkaloid production from *in-vitro* grown plantlets in comparison to field-grown plants.

MATERIALS AND METHODS

Present study was carried out in Department of Plant Physiology, G B Pant University of Agriculture and Technology, Pantnagar during 2008–10. The explants, ie leaves, apical and nodal tissues were obtained from field grown plants in Medicinal Research and Development Centre, G B Pant University of Agriculture and Technology, Pantnagar. For both direct and indirect organogenesis, the explants were thoroughly washed with Tween 20 (1.0%), thereafter, for 10 min. with bavistin (6% w/v) and finally rinsed with double distilled water. The explants were then surface sterilized for 30 sec. in ethanol (70%), followed by sodium hypochlorite (1% v/v) for 1 min. and then with mercuric chloride (0.1% w/v) for 1 min. and finally rinsed five times with double distilled water. To remove moisture the explants were then placed upon sterile filter papers.

Square leaf discs (1.0 cm^2) and nodal segments (10 mm) were inoculated on MS medium containing sucrose (30 g/litre) supplemented with various combinations of growth hormones, i e 2, 4-D (2, 4-dichlorophenoxyacetic acid, 1 to 2 mg/litre), BAP (6-benzyl amino purine, 1 mg/litre), NAA (α-Naphthalene acetic acid, 2 mg/litre), IAA (indole-3 acetic acid, 2 mg/litre) and solidified with 8 g/litre agar to study the callogenic response. For direct organogenesis shoot apices (8 mm) and nodal segments (10 mm) were inoculated on MS medium containing sucrose (30 g/litre) supplemented with various combinations of growth hormones i.e., BAP (1 to 1.5 mg/litre), NAA (0.1 to 0.5 mg/litre), IAA (0.1 to 0.5 mg/litre), KIN (kinetin, 1.0 mg/litre), GA_3 (gibberellic acid, 0.1 mg/litre) and solidified with 8 g/litre agar. The cultures were incubated at 25 ± 2ºC with 16 hr light and 8 hr/dark with 60 μmol/m^2 s light intensity provided by cool white fluorescent tubes (40 W; Phillips, India). The relative humidity within culture room was maintained at 60 per cent. In order to analyze the critical concentration which can prevent contamination arising due to endophytic microflora, different concentrations of bavistin (contains 50% carbendazim w/w) and streptomycin were supplemented in all growth mediums and per cent contamination was recorded. For regeneration, two-week old calli were sub-cultured on MS medium supplemented with various combinations of growth hormones, ie BAP, NAA, IAA, KIN and GA_3. Two weeks old regenerated plantlets obtained through both direct and indirect organogenesis were transferred on to MS + BAP (1 mg/litre) + NAA (0.1 mg/litre) for multiple shoot proliferation. Shoot proliferation was evaluated in terms of average shoot length (cm) and number of shoots per regenerant. Six-week old regenerated shoots developed both through direct or indirect organogenesis were placed onto rooting medium containing MS + IBA (1.0 mg/litre) + IAA (1.0 mg/litre). Well rooted plants were transplanted into pots containing autoclaved soil mixture and vermiculite (3:1 v/v). The plantlets were grown under humid conditions in a greenhouse at temperature 28±2°C up to maturity.

Total alkaloid content was extracted from *in vitro*-grown plantlets as described earlier (Batista *et al.* 1996) and expressed on per cent dry weight basis. For the extraction of total alkaloid content, 5g dry matter of the plants or plant parts were taken from six-week old multiple shoots and one-year old field-grown plant for comparison.

The experiments were set up in a completely randomized block design (CRD) with each treatment replicated four times to estimate the average values. The analysis was carried out with SPS software (Department of Statistics, G B Pant University of Agriculture and Technology, Pantnagar, Uttarakhand)

RESULTS AND DISCUSSION

Effect of plant growth regulators on callus induction

For callus induction during indirect organogenesis using leaves and nodal segments as explants, seven different combinations of hormones, namely 2, 4-D, BAP, NAA and IAA were applied (Table 1). Light green and friable calli

Table 1 *In vitro* response of different plant hormones in MS medium on callus induction with leaf and nodal segments as explants source

MS medium + hormone (mg/litre)	Callus induction response	
	Leaf	Nodal segment
2, 4-D (2.0)	Yellowish green and friable	No response
2, 4-D (2.0) + BAP (1.0)	Light green and friable	Yellowish green and friable
2, 4-D (1.0) + BAP (1.0)	Yellowish and compact	Light green and compact
NAA (2.0)	No response	Yellowish and compact
NAA (2.0) + BAP (1.0)	Light green and compact	Yellowish green and compact
IAA (2.0)	No response	No response
IAA (2.0) + BAP (1.0)	No response	Brown, friable and loose

Fig 1 Primary callus induction and direct organogenesis from different parts of *R. serpentina* (*A*) Primary callus induction on MS medium from the leaf tissue, (*B*) regeneration of shoot from unorganized callus, (*C*) direct regeneration from the shoot apices, (*D*) direct regeneration from the nodal segment, (*E, F*) multiple shoot culture from direct and indirect organogenesis in MS + BAP (1 mg/ litre) + NAA (0.1 mg/litre), (*G*) root induction in MS + IBA (1.0 mg/ litre) + IAA (1.0 mg/ litre), (*H*) normal plantlet regenerated through organogenesis in greenhouse

were observed in MS + 2.0 mg/litre 2, 4-D + 1.0 mg/litre BAP when leaves were taken as explant. When nodal segments were taken as explant, yellowish green and friable calli were observed in MS + 2.0 mg/litre 2, 4-D + 1.0 m/litre BAP (Fig 1A). Cytokinin in combination with auxin using various explants for callus induction will be necessary for callus growth and higher auxin concentration may be required for callus initiation as reported earlier by several authors (Jia *et al.* 2008, Kumar *et al.* 2011).

Endophytic microflora and bacterial contamination are major problems during callus induction in *R. serpentina* (Sarma *et al.* 1999). To check this, bavistin (6.0 g/litre) and streptomycin (100 mg/litre) was found most effective (Fig 2 A, B). Bavistin was reported earlier as a systemic fungicide which can enter the tissue system and kill the endophytic microflora present inside the explant. However, higher concentration of bavistin can also adversely affect the tissue (Nene and Thapliyal 2002) similar to our experiment.

Effect of plant growth regulators on plant regeneration

Well proliferated two-week old calli were transferred onto MS medium supplemented with 12 different combinations of hormones, namely BAP, NAA, IAA, KIN and GA_3 for shoot bud differentiation from calli (Table 2). The highest shoot morphogenesis and regeneration was obtained when calli were subcultured on MS + 1.0 mg/litre BAP + 0.1 mg/litre KIN, followed by MS + 1.0 mg/litre BAP + 0.1 mg/litre KIN + 0.1 mg/litre GA_3 (Fig 1B). However, no regeneration was observed in MS + 1.0 mg/litre IAA and MS + 1.0 mg/litre IAA + 0.1 mg/litre GA_3. Shoot regeneration via callus phase during indirect organogenesis were described in several medicinal plants. Furthermore, a variety of results from different plant species suggest that GA_3 stimulate numerous GAST-like (Gibberellic Acid Stimulated Transcript) genes which are mainly involved in cell elongation and cell division. GA_3 also induce the synthesis of photosynthetic pigments in developing calli and thus improve photosynthetic

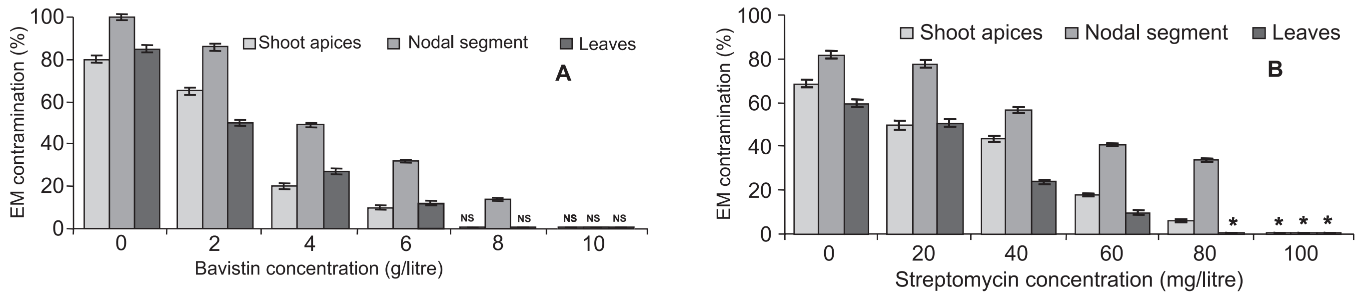

Fig 2 Effect on per cent fungal contamination due to endophytic microflora (EM) in different explants under different concentrations of (*A*) Bavistin (g/ litre), and (*B*) streptomycin (mg/ litre) supplemented in MS media. Data represents mean ± SE of four replications of each explant for each treatment. NS indicates no tissue survival and * indicates complete removal of contamination

Table 2 *In vitro* response of different combinations of plant hormones supplemented with MS medium on indirect organogenesis and plantlet differentiation from callus

MS medium + hormone (mg/ litre)					Morphogenic response
BAP	KIN	GA_3	NAA	IAA	
1.0	0.1	0.1	0.0	0.0	Multiple shoot with good growth
1.0	0.1	0.0	0.0	0.0	Multiple shoot with good growth
1.5	0.1	0.0	0.0	0.0	Multiple shoot with poor growth
1.0	0.0	0.1	0.1	0.0	Single shoot with good growth
1.0	0.0	0.0	0.1	0.0	Single shoot with poor growth
1.5	0.0	0.0	0.1	0.0	Single shoot with good growth
1.0	0.0	0.0	0.5	0.0	Single shoot with poor growth
1.5	0.0	0.0	0.5	0.0	Slow growth
1.0	0.5	0.0	0.0	0.0	Slow growth
1.5	0.5	0.0	0.0	0.0	Slow growth
0.0	0.0	0.0	0.0	1.0	No response
0.0	0.0	0.1	0.0	1.0	No response

ability, extra storage reserves and carbohydrates favour *in vitro* germination (Joshi *et al.* 2010).

For direct regeneration explants (apical and nodal segments) were inoculated on MS medium supplemented with 12 different combinations of hormones, namely BAP, NAA, IAA, KIN and GA_3 (Table 3). Maximum response for multiple shoot induction was observed when explants were inoculated on MS + 1.0 mg/litre BAP + 0.1 mg/litre KIN, followed by MS + 1.0 mg/litre BAP + 0.1 mg/litre KIN + 0.1 mg/litre GA_3 (Figs 1C, D). However, no regeneration was observed in MS + 1.0 mg/litre IAA and MS + 1.0 mg/litre IAA + 0.1 mg/litre GA_3 similar to indirect organogenesis. Cytokinins, especially BAP, were reported to overcome apical dominance, release lateral buds from dormancy and promote shoot formation (Shivaraj and Rao 2011). Effect of BAP along with KIN and GA_3 leading to cell differentiation, elongation, floral bud formation and maturation was confirmed by previous authors (Bhatt *et al.* 2008, Fadel *et al.* 2010).

Table 3 *In vitro* response of different combinations of plant hormones supplemented with MS medium on direct organogenesis from nodal segment and shoot apices taken as explants

Explant source	MS medium + hormone (mg/ litre)					Morphogenic response
	BAP	KIN	NAA	G.A.	IAA	
Shoot apices	1.0	0.1	0.0	0.0	0.0	Multiple shoot with good growth
	1.0	0.5	0.0	0.0	0.0	Poor growth
	1.0	0.1	0.0	0.1	0.0	Multiple shoot with good growth
	1.5	0.1	0.0	0.0	0.0	Single shoot with good growth
	1.5	0.5	0.0	0.0	0.0	Poor growth
	1.0	0.0	0.1	0.0	0.0	Single shoot with good growth
	1.0	0.0	0.1	0.1	0.0	Single shoot with good growth
	1.0	0.0	0.5	0.0	0.0	Poor growth
	1.5	0.0	0.1	0.0	0.0	Poor growth
	1.5	0.0	0.5	0.0	0.0	Poor growth
	0.0	0.0	0.0	0.1	1.0	No response
	0.0	0.0	0.0	0.0	1.0	No response
Nodal segment	1.0	0.1	0.0	0.1	0.0	Multiple shoot with good growth
	1.0	0.1	0.0	0.0	0.0	Single shoot with good growth
	1.0	0.5	0.0	0.0	0.0	Single shoot with good growth
	1.5	0.1	0.0	0.0	0.0	Single shoot with good growth
	1.5	0.5	0.0	0.0	0.0	Poor growth
	1.0	0.0	0.1	0.1	0.0	Single shoot with good growth
	1.0	0.0	0.1	0.0	0.0	Poor growth
	1.0	0.0	0.5	0.0	0.0	Poor growth
	1.5	0.0	0.1	0.0	0.0	Poor growth
	1.5	0.0	0.5	0.0	0.0	Poor growth
	0.0	0.0	0.0	0.1	1.0	Poor growth
	0.0	0.0	0.0	0.0	1.0	No response

Proliferation of plantlets and hardening

For multiple shoot proliferation two-week old regenerated plantlets were transferred on to MS + BAP (1 mg/litre) + NAA (0.1 mg/litre) (Fig 1 E, F). During the initial two weeks of subculture, explants did not show any significant variation in number of shoots (Fig 3 A), leaf (Fig 3 B) and plant height (Fig 3 C). However, effective variation in all three traits was observed after two weeks. Maximum number of leaves, shoots and height were observed with shoot tips as explants source. BAP as a cytokinin was reported to be most efficient in comparison to KIN, zeatin, 2-isopentenyladenine or thidiazuron for axillary bud initiation and subsequent proliferation (Muñoz-Concha and Davey 2010). However, addition of auxin at low concentration significantly enhanced shoot proliferation and was reported to be very important for mass scale propagation (Salma *et al.* 2008). Production of multiple shoots with BAP showed the confirmation of earlier work in *Rauwolfia* (Anitha and Kumari 2006).

Well proliferated multiple shoots were transferred to rooting medium, MS + IBA (1.0 mg/litre) + IAA (1.0 mg/litre) and after two weeks, profuse rooting was achieved (Fig 1 G). Effective root induction was reported earlier on MS medium supplemented with IAA and IBA (Ahamed *et al.* 2005, Salma *et al.* 2008). Proliferated, well rooted plants were transferred into pots (Fig 1H) and grown successfully in greenhouse up to maturity. The regenerated plants were successfully established in the pots under field conditions.

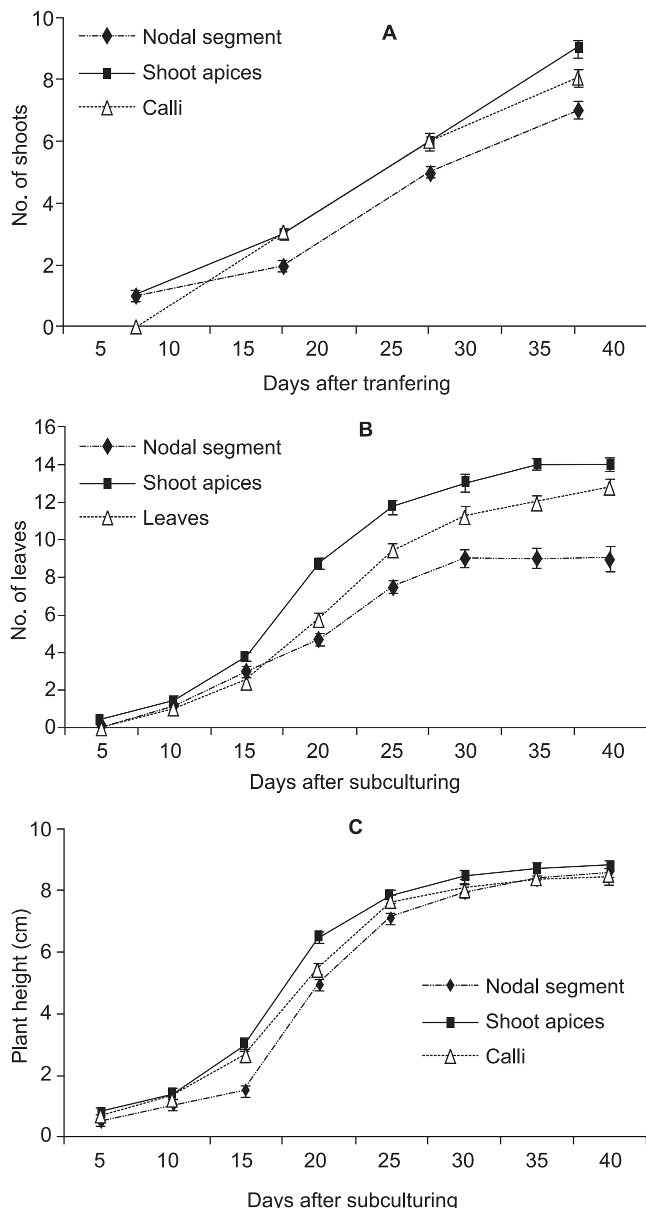

Fig 3 Average number of shoots produced during direct and indirect organogenesis of *R. serpentina* in MS + BAP (1 mg/ litre) + NAA (0.1 mg/litre) (*A*) Average number of leaves produced during direct and indirect organogenesis of *R. serpentina* in MS + BAP (1 mg/ litre) + NAA (0.1 mg/litre) (*B*) average plant height during direct and indirect organogenesis of *R. serpentina* in MS + BAP (1 mg/ litre) + NAA (0.1 mg/litre) (*C*) Data represent the mean ± SE of four replicates

Total alkaloid extraction

Six-week old multiple shoots were taken for the extraction of total alkaloid content and compared with different plant parts of one-year old field-grown plant (Fig 4). Maximum total alkaloid content (0.321% D.W.) was found in roots of field-grown plant is much higher than obtained through *in vitro*-grown multiple shoot cultures. However, total alkaloid content of *in vitro* developed multiple shoots (0.0786% D W) was similar to that of average total alkaloid content obtained through the leaves (0.0526% D W) and stem (0.0960% D W) of one-year old field-grown plants. It was earlier reported that the whole plant of *Rauwolfia serpentina* is rich in alkaloids (Baskarrajan *et al.* 2003). The higher concentration of alkaloids present in the *in vitro* developed plantlets as compared to that of leaves of field-grown plants is probably due to higher cell to cell contact, ageing and limited differentiation of the cells during differentiation in the culture. It was also reported that alkaloid

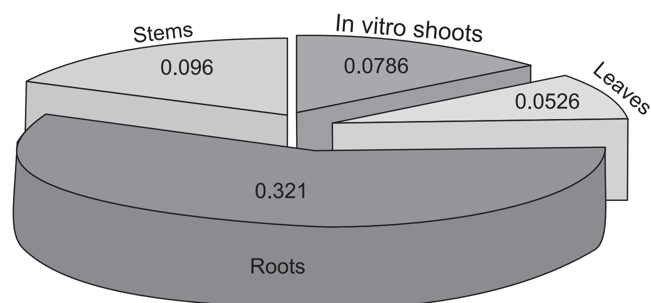

Fig 4 Comparative account of total alkaloid content (% DW) in leaves, stem and roots of one-year old field-grown plant and *in vitro* developed multiple shoots of *Rauwolfia serpentina*

biosynthetic capacity increased upon shoot differentiation (Paranhos *et al.* 2005).

Considering the complex spatial and temporal regulation of alkaloid accumulation, it is practical that appropriate management of alkaloid producing plants can significantly increase bioactive product yields at very low cost. However metabolic engineering and physiological manipulation can be used as an alternative to improve the alkaloid yield (Pasquali *et al.* 2006).

Previous *in vitro* studies on this plant were mainly focused upon isolation and optimization of alkaloids through callus culture. Here, we have reported a reproducible and efficient direct and indirect organogenesis of *R. serpentina* that can serve as a promising source of alkaloid extraction in a comparatively lesser time. Total crude alkaloid production from multiple shoot culture shows immense potential to use it at large scale for massive propagation towards commercialization of many MIAs.

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