



Effect of growth regulators on micropropagation of *Rauvolfia serpentina* (L.) Benth

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Abstract: An efficient protocol for micropropagation through *in vitro* culture of *Rauvolfia serpentina* was standardized. Out of different combination of phytohormone tested, MS media supplemented with 0.5 mg L⁻¹ Indole Acetic Acid + 0.5 mg L Nephthalene acetic acid was found to be finest for mean callus induction (62.66%) as well as callus mediated shoot regeneration with mean percentage response (56) and number of shoot per culture (5). In direct shoot regeneration, best growth of axillary shoots was obtained on MS media supplemented with 0.5 mg L⁻¹ Indole Acetic Acid + 0.5 mg L⁻¹ Benzyl Amino Purine with maximum mean percentage response(77.33) and number of shoots per culture (9.0) ,however the best shoot elongation of shoot was found on MS media supplemented with 3.0 mg L⁻¹ IAA plus 3.0 mg L⁻¹BAP with 6.50(mean) . Higher induction of root (88%) with mean number of root per culture (12) was observed in MS medium supplemented with Indole Butyric Acid (3.0 mg L⁻¹). The rooted plantlets were successfully established in the field. The protocol was optimized by manipulations of different PGRs for enhanced multiplication. Protocol explained in this research paper provides a rapid plant regeneration system which could be used for production of large number of true to the type, uniform, disease free, elite, plantlets right through the year, which will make things easier for large scale cultivation of this endangered important medicinal plant.

Keywords: Growth regulators, Medicinal plant, Micropropagation, *Rauvolfia serpentina*

INTRODUCTION

Rauvolfia serpentina (L.) Benth. Ex Kurz. (Apocynaceae) a woody perennial shrub, commonly known as 'Sarggandha' is an endangered medicinal plant found in Bangladesh, China, Indonesia, India, Malaysia, Nepal, Pakistan, Sri Lanka and Viet Nam (Dey and De, 2010; and Sushila *et al.*, 2013). The roots of this shrub have been used for centuries in ayurvedic medicines. Root are bitter, acrid, laxative, thermogenic, diuretic and possess sedative properties. This herbal plant is used as medicine for high blood pressure, insomnia, anxiety and other disorders of the central epilepsy. It is highly reputed for hypertension and is useful in stangury, fever, wounds, insomnia, epilepsy and dyspepsia, remedy for the removal of opacities of the cornea, increase uterine contractions and anthelmintic and antidote to snake venom (Prakash, 2001; Jain *et al.*, 2003 and Pawar *et al.*, 2011).

Reserpine is a potent indole alkaloid first isolated from this plant which is being widely used as an antihypertensive (Anonymous, 2003). Due to poor seed germination rate, large scale and indiscriminate collection of wild plants for pharmaceutical purposes

and insufficient attempts to either allow its replenishment or its cultivation, *R. serpentina* is rapidly disappearing and is now listed as an endangered species by the International Union for Conservation of Nature and Natural Resources (IUCN) (Jain *et al.*, 2003; Singh *et al.*, 2009; Sushila *et al.*, 2013). The propagation of *R. serpentina* through seeds is difficult due to less viability and very low germination percentage (25% - 50%) and is often as low as 10 percent that is due to the presence of cinnamic acid derivatives (Mitra, 1976; Mallik *et al.*, 2012). *In vitro* culture is an alternative method for conservation and propagation of this species. Propagation of *R. serpentina* through tissue culture has previously been described but most of them have focused on micro propagation by means of stimulating auxiliary shoot from shoot tip as explants (Singh, 2009; Harisaranraj, 2010 and Rani *et al.*, 2013). However, detailed holistic study on *in vitro* regeneration of *R. serpentina* in Bihar is lacking. Therefore, this study was aimed at development of an efficient protocol for micropropagation of *R. serpentina* by optimizing growth regulators such as auxin and cytokinins.

MATERIALS AND METHODS

Plant material: The shoots of about 1.5-2 cm long were collected from the experimental garden of Department of Agricultural Biotechnology and Molecular Biology at RAU, Pusa (Bihar) for *in vitro* studies in year 2009-2010. The explants were first washed with running tap water for 20 min., soaked in 70% (v/v) ethanol for 1min., surface sterilized with Tween-20 (6 drop L⁻¹) for 20 min. and rinsed in sterile distilled water thrice. The explants were then treated with 0.1% HgCl₂ for 2 min. and washed with sterile distilled water in laminar air flow chamber.

Culture medium and culture conditions: The explant was then inoculated in the appropriate MS medium (Murashige and Skoog, 1962), which contained sucrose (3%), and pH (5.8). Cutting edge of the explants was kept in direct contact with the medium. The cultures were incubated at 24±2°C under 2000 lux light intensity using white fluorescent lamp for 16 hr photoperiod. The basal MS medium was used with derived supplementation of phyto-regulators for the induction of shoots and roots. The MS basal media supplemented with different auxins such as The MS basal media supplemented with different auxins: Indole Butyric acid (IBA) 0.5, 1.0, 1.5, 2.0, 3.0 mgL⁻¹, Indole Acetic acid (IAA) 0.5, 1.0, 2.0, 3.0 mg L⁻¹ and Naphthalene Acetic Acid (NAA) 0.5, 1.0, 1.5, 2.0 mgL⁻¹ and cytokinin: Benzyl Amino Purine (BAP) 0.5, 1.0, 1.5, 2.0, 3.0 mgL⁻¹ and Kinetin (KIN) 0.5, 1.0 mg L⁻¹. At this culturing stage, various types and concentrations of plant growth regulators (PGRs) were evaluated for callusing, shoot proliferation and root formation.

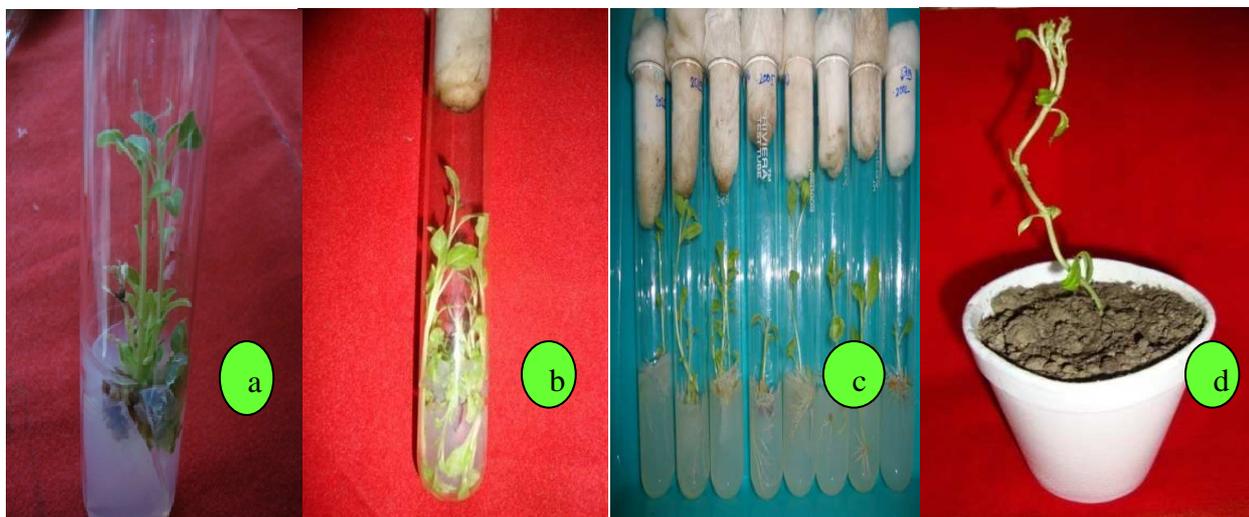
Acclimatization: The rooted shoots were removed from the culture tube and washed with sterile water to the remove any traces of agar on the roots and dipped in 0.2% (w/v) carbendazim 50 WP (Bavistin) solution for 20 min. Plantlets were transferred to plastic pots

filled with sterile soil and kept in green house. Initially, the pots were covered with polybags and after 25 days these were uncovered and shifted gradually from shade to sunlight.

Statistical analysis: Each *in vitro* experiment was conducted in completely randomized design and each treatment replicated thrice with 20 explants per treatment. The frequency of callogenesis, shoot formation on callus, direct shoot formation and root regeneration per culture were determined upto 50 days after inoculating explants on different media. The data was analyzed by using the OPSTAT statistical package software.

RESULTS AND DISCUSSION

Callogenesis: Aseptic shoot apex explants of *R. serpentina* were cultured aseptically under *in vitro* condition. After a week, callus induction and proliferation were obtained by inoculating sterile shoot apex only on MS medium containing 3.0% sucrose (w/v) which supplemented with different concentrations of BAP (0.5,1.0,1.5 mg L⁻¹) with KIN (0.5,1.0 mg L⁻¹) or NAA (0.5, 1.0mg L⁻¹). The rate of callus formation was slow in the first two weeks. However, callus exhibited good growth and covered the whole explant within 3 - 4 weeks. Shoot primordia also developed on the callus, which exposed its organogenic nature. The major portion of the callus was soft friable and creamy white. Out of different combination of phytohormone tested, 0.5 mg L⁻¹ NAA+ 0.5 mg L⁻¹BAP was found to be best for mean callus induction (62.66) percentage and days to callus induction (Plate 1). The combination showed 53% response with respect to the callus induction in shoot apex in 28 days. The callus induction gradually reduces with increased concentration of BAP (Table 1). Adhikari and Pant (2013) reported that the best callusing were observed on MS media supplemented with BAP 0.5 mg L⁻¹ + NAA 1.5 mg L⁻¹ in *W.somnifera*. Mallik *et. al.*, 2012 obtained good callus



Plates 1. *In vitro* responses from shoot apex in genotype RS-1 of *R. serpentina* (a) Callus mediated shoot induction; (b) Direct shoots proliferation; (c) Rooting of regenerated shoots; (d) Regenerated plantlets in pot.

growth on MS medium on *Rauwolfia serpentina* stem explants using a combination of BAP (1.0 - 2.5 mg L⁻¹) and IAA (0.1 - 0.2 mg L⁻¹) which is contrary to our result. We did not get callus on MS media on shoot apex explants using a combination of BAP (0.5, 1.0, 2.0, 3.0) and IAA(0.5, 1.0, 2.0, 3.0). However, poor growth of callus is observed on MS media on shoot apex explants using a combination of BAP(0.5,1.0) and KIN(0.5,1.0) .

Shoot regeneration on callus: The callus derived from shoot apex explants produce multiple shoots after 2-4 weeks of culture in basal MS medium which supplemented with different concentrations of BAP (0.5, 1.0, 1.5 mg L⁻¹) with KIN (0.5, 1.0 mg L⁻¹) or NAA (0.5, 1.0mg L⁻¹). Callus mediated shoot proliferation and elongation were observed on MS medium supplemented with varying concentrations of BAP or KIN (Table 1). At higher concentrations of BAP or KIN, the growth and no. of the shoots per culture was lower (Plate 1a). The MS Medium containing BAP (0.5 mg L⁻¹) plus KIN (0.5 mg L⁻¹) was observed as the most effective combination for callus mediated shoot induction resulted better mean number of shoots per culture (4.0) with mean shoot length (3.0 cm). Panwar *et al.* (2011) reported that at higher concentration of BAP the number of shoots per culture declined gradually in the callus derived from leaf explants of *R. serpentina* as found in our result. Harisaranraj *et al.* (2011) reported that at higher

concentration of either NAA or BAP in the culture MS medium inhibited the growth of the shoots and stimulated small callusing at the basal end of *R. serpentina* as in our observation (Table 1, Plate-1). The number of multiple shoots per explants varied from 2.00 to 5.00 between treatments with percentage response (16-73). Edson *et al.*, 1996 reported that minimal cytokinins and auxins in culture media would avoid somaclonal variation and efficiently produce true to type plantlets.

Direct shoot regeneration: The *in vitro* multiplication of *R. serpentina* shoots through the sprouting of axillary buds is the most commercially viable means of micropropagation. The initial response of axillary shoot buds formation was observed after 2-3 weeks of culturing of shoot apex explant (Table 1). Out of the fourteen combinations of shoot induction media tested, direct shoot regeneration was obtained only on seven MS media which contained different concentrations of IAA and BAP. A progressive degree of percentage response and no. of axillary shoot bud per culture were observed with decrease the concentration of IAA with BAP while, at higher concentrations led to increase in shoot elongation. The maximum mean number of shoots per culture (9.00) and mean percentage response (77.33cm) was obtained on MS media supplemented with 0.5 mg L⁻¹ IAA plus 0.5 mg L⁻¹ BAP. (Table 1, Plate 1(b)). The best response of axillary shoot bud elongation was observed with

Table 1. *In vitro* response from shoot apex explants of *R. serpentina* cultured on different compositions of (IAA, NAA, BAP and KIN) MS media.

Phytohormone in (mg L ⁻¹)				Response (%)	Mean No. of shoots/culture (Mean±SE)	Mean length of shoot in cm (Mean±SE)	Mean induction of callus (Mean±S)	Nature of callus
IAA	BAP	KIN	NAA					
	0.5	0.5		52.66±0.88	4.00±0.57	3.00±0.57	37.66±1.45	F,W
	1.0	0.5		59.00±1.15	3.00±0.57	1.00±0.28	26.66±0.88	SF,W
	1.0	1.0		56.00±0.57	3.66±0.33	0.70±0.05	13.00±1.00	SF,W
	0.5		0.5	56.00±1.15	5.00±1.15	2.50±0.76	62.66±1.45	SF,W
	0.5		1.0	16.00±0.57	4.33±1.20	3.66±1.20	53.00±1.73	SF,W
	1.0		1.0	36.66±0.88	2.00±0.57	2.66±0.88	43.66±1.66	SF,W
	1.5		0.5	73.00±1.15	2.33±0.33	3.33±0.66	25.33± 1.45	SF,W
0.5	0.5			77.33±1.20	9.00±0.57	3.33±0.33	-	-
1.0	1.0			46.66±0.88	6.00±0.57	4.66±0.33	-	-
1.0	2.0			53.66±0.33	4.33±0.33	4.66±0.66	-	-
2.0	3.0			47.33±0.88	2.33±0.33	5.00±0.57	-	-
3.0	1.0			13.33±0.88	2.66±0.66	6.43±0.29	-	-
3.0	2.0			24.66±1.45	4.66±0.33	5.70±0.17	-	-
3.0	3.0			27.00±0.57	3.00±0.57	6.50±0.36	-	-
C.D. (<i>p</i> ≤ 0.05)				2.75	1.87	1.72	2.90	
S.E.(m)				0.94	0.64	0.59	0.99	

Visual observation; callus - = no response; W = white; F = friable; SF = semifriable

Table 2. *In vitro* response from regenerated shoots explants of *R. serpentina* subcultured on different compositions of (IBA and NAA) MS media for rooting.

Phyto hormone in (mg L ⁻¹)		Response (%)	Mean No. of root/shoots (Mean±SE)	Mean length of root in cm (Mean±SE)
IBA	NAA			
0.5		73.00±3.00	7.33±1.76	4.46±0.88
1.0		74.00±3.05	3.33±0.66	3.66±0.88
1.5		75.66±2.18	3.33±0.88	3.66±0.33
2.0		71.66±3.28	2.66±0.88	2.00±0.57
3.0		88.00±1.52	12.00±1.73	6.33±0.33
	0.5	84.66±1.45	8.66±1.85	6.00±1.15
	1.0	84.66±2.60	11.33±3.52	3.33±0.66
	1.5	87.66±1.76	5.00±1.15	2.66±0.66
	2.0	87.66±1.20	3.66±1.45	1.66±0.33
C.D. ($p \leq 0.05$)		7.03	5.22	2.10
S.E.(m)		2.349	1.74	0.70

6.50(mean) on MS media supplemented with 3.0 mg L⁻¹ IAA plus 3.0 mg L⁻¹ BAP. Thus, the propagation of plants from axillary buds has proved to be the most generally applicable and reliable method of *in vitro* propagation in *R. serpentina*, as the regeneration of the plant is very difficult from seeds and other sources. The seeds are mostly non-viable due to abortive embryos (Mitra, 1976; Akram and Illahi, 1986). Harisaranraj *et al.* (2010) reported that the growth of the shoots was stunted with increase in the frequency of concentrations of BAP, NAA or KIN in *R. serpentina* which is in consent with present result.

Root regeneration and acclimatization: Rooting ability of *in vitro* regenerated shoots in the present studies was satisfactory. *In vitro* regenerated shoots produced profuse rooting after 15-20 days of sub culturing on MS medium supplemented with various concentrations of NAA or IBA. Out of different hormone concentration of MS media tested, IBA (3.0 mg L⁻¹) was found to be best for root induction percentage (88 %), mean number of root per explant (12) and mean root length (6.33 cm) followed by NAA (1.0 mg L⁻¹) with root induction percentage (84.66 %), mean number of root per explant (11.33) and mean root length (3.3 cm). The roots were long, pale white, robust with root hairs (Table 2). Plate 1 (c). The present result is in accordance with the result reported by Rahman *et al.* 2008. They reported that the adventitious shoots were best rooted on half strength MS medium supplemented with 1.0 mg L⁻¹ of IBA. NAA and IBA has been reported to have a stimulatory effect on root induction in many tree species (Channd, 1995). Susila *et al.* (2013) got 100% rooting when transferred *in vitro* regenerated multiple shoots of *R. serpentina* on half-strength MS medium supplemented with NAA (0.2 mg L⁻¹) plus IBA (0.2 mg L⁻¹). Finally, the developed plantlets were kept in poly-house and greenhouse for hardening, and then

transferred to field. About 70% of the plants transferred to field survived and did not show any detectable variation in morphology or growth characteristics when compared to the mother plants (Plate 1e).

In conclusion, an efficient protocol for micropropagation of an important medicinal plant, *R. serpentina*, was developed in this study by testing various concentrations of growth regulators and culture conditions. This method produces a large number of true to the type, uniform, disease free, elite, plantlets right through the year, which will make things easier for large scale cultivation of this endangered important medicinal plant for commercial extraction of alkaloid for therapeutic use. This can lead to the synthesis and extraction of active compounds from its root.

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