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Short Communication

MICROPROPAGATION OF SERPGANDHA (*Rauvolfia serpentina* L. Benth): AN ENDANGERED MEDICINAL PLANT

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ABSTRACT

An efficient protocol for propagation of *Rauvolfia serpentina* through *in vitro* culture was standardized. Of the various combinations of phyto-hormones evaluated, 0.5 mg L^{-1} indole acetic acid (IAA) + 0.5 mg L^{-1} benzyl amino purine (BAP) + 1.0 mg L^{-1} kinetin highly favoured callus induction (32.8%). High callus-mediated shoot regeneration was seen on MS medium supplemented with 1.0 mg L^{-1} IAA + 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} kinetin. Low auxin (IAA, 0.5 mg L^{-1}) with higher concentration of cytokinin (0.5 mg L^{-1} BAP + 1.0 mg L^{-1} kinetin) gave higher shoots culture^{-1} (6.6) with mean shoot length of 3.2 cm. In direct shoot regeneration, best growth of axillary shoots was achieved in MS medium supplemented with 0.2 mg L^{-1} IAA + 0.2 mg L^{-1} BAP + 0.2 mg L^{-1} kinetin with maximum shoots culture^{-1} (10.0) and shoot length (4.4 cm). The root induction was highest (84.3%) in MS medium supplemented with IBA (0.5 mg L^{-1}). The rooted plantlets were successfully established in field.

Key words: Endangered species, *in vitro* propagation; medicinal plant, *Rauvolfia serpentine*

INTRODUCTION

Rauvolfia serpentina (L.) Benth. Ex. Kunz (family: Apocynaceae) is a woody perennial shrub, commonly known as sarpagandha, snake root plant, chotachand, chandrika, etc. The roots of this shrub are used in ayurvedic medicines for centuries. The plant is native to Indian sub-continent and East Asia (Sushila *et al.*, 2013). The plant contains about 50 indole alkaloids (0.7-2.4%), phyto-sterols, unsaturated alcohols and sugars. Among the various alkaloids, reserpine, recinnamine, serpentine and ajamaline are the active constituents well-known for anti-hypertensive action (Panwar *et al.*, 2011). *R. serpentina* roots are bitter, acrid, laxative, thermogenic, diuretic and possess sedative properties. It is highly reputed for as remedy for hypertension as well as used against stangury, fever, wounds, insomnia, epilepsy, dyspepsia, cornea opacity, uterine contractions and anthelmintic and antidote to snake venom (Jain *et al.*, 2003; Panwar *et al.*, 2011). Due to indiscriminate and ruthless harvesting, *R. serpentina* has been included in "red" listed plants of India (Singh *et al.*, 2009; Sushila *et al.*, 2013). The natural habitat of this shrub is dwindling fast due to the increasing anthropogenic activities and over-extraction of this plant for pharmaceutical purpose (Singh *et al.*, 2009; Panwar *et al.*, 2011). The propagation of *R. serpentina* through seeds is

difficult due to less viability and very low germination, often as low as 10% due to the presence of cinnamic acid derivatives (Mallick *et al.*, 2012). To cope up this alarming situation and enhance mass multiplication of *R. serpentina*, there is urgent need to develop *in vitro* multiplication techniques. Direct regeneration of *R. serpentina* from shoot apex has been reported earlier (Ahmad *et al.*, 2002). However, regeneration using shoot apex explants derived from RS-1 genotype of sarpgandha is unavailable. The genotype 'RS-1' is a high root yield producing genotypes but has poor seed germination. Hence, the present study was aimed to develop an efficient protocol for micropropagation of *Rauvolfia serpentina* by optimizing growth regulators for *in situ* and *ex situ* plantation.

MATERIALS AND METHODS

The seeds of sarpgandha (*R. serpentina*) genotype RS-1 were collected from JNKVV, Jabalpur (MP) and established in the experimental garden of Department of Agricultural Biotechnology and Molecular Biology at RAU, Pusa (Bihar). The shoots (1.5-2.0 cm long) were collected at 9 a.m. from apex of 2 years old plants 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% $HgCl_2$ for 2 min. and washed with sterile distilled water in laminar air flow chamber. It was then inoculated in the appropriate MS medium (Murashige and Skoog, 1962), which contained sucrose (3%), and pH (5.8). Cutting edge of explants was kept in direct contact with the medium. The cultures were incubated at 24±2ºC 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 indole butyric acid (IBA) 0.5-4.0 mg L^{-1}, indole acetic acid (IAA) 0.2-2.0 mg L^{-1} and naphthalene acetic acid (NAA) 2.0-4.0 mg L^{-1} and cytokinin such as benzyl amino purine (BAP) 0.2-1.0 mg L^{-1} and kinetin (Kn) 0.2-1.0 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. 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. Each *in vitro* experiment was conducted in a 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

Shoot apex explants of *Rauvolfia serpentina* were cultured aseptically under *in vitro* conditions. MS medium containing 3.0% sucrose (w/v) and various plant growth regulators like IAA, BAP and Kn were tested for shoot initiation (Table 1). After one week, a shinny cream coloured callus was induced on the explants only on MS media supplemented with either 1.0 mg L^{-1} IAA + 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} Kn or 0.5 mg L^{-1} IAA+0.5 mg L^{-1} BAP + 1.0 mg L^{-1} Kn. The rate of callus formation was slow in the first 2 weeks. However, callus exhibited good growth and covered the

Table 1: *In vitro* response from shoot apex explants of *R. serpentina* cultured on MS medium supplemented with various phytohormones

Phytohormone (mg L^{-1})			Response (%)	Shoots culture^{-1} (No.) (Mean ± SE)	Shoot length (cm) (Mean ± SE)	Callus induction (Mean ± SE)	Nature of Callus
IAA	BAP	Kn					
0.2	0.2	0.2	80.1 ± 0.55	10.06 ± 0.35	4.49 ± 0.57	-	-
0.5	0.5	0.5	78.5 ± 0.17	7.66 ± 1.45	4.13 ± 0.54	-	-
1.0	1.0	1.0	71.0 ± 0.57	5.00 ± 0.57	1.50 ± 0.28	-	-
1.0	1.0	0.5	68.6 ± 1.02	6.33 ± 0.88	0.93 ± 0.29	-	-
1.0	0.5	1.0	56.9 ± 0.43	6.00 ± 0.57	2.73 ± 0.14	-	-
1.0	0.5	0.5	53.0 ± 0.50	5.00 ± 0.57	2.03 ± 0.29	19.06 ± 0.67	SF, W
0.5	0.5	1.0	47.2 ± 1.26	6.66 ± 2.40	3.23 ± 0.37	32.86 ± 1.50	SF, W
SE(m)±			0.67	0.96	0.40		
$CD_{0.05}$			2.11	3.01	1.25	-	-

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

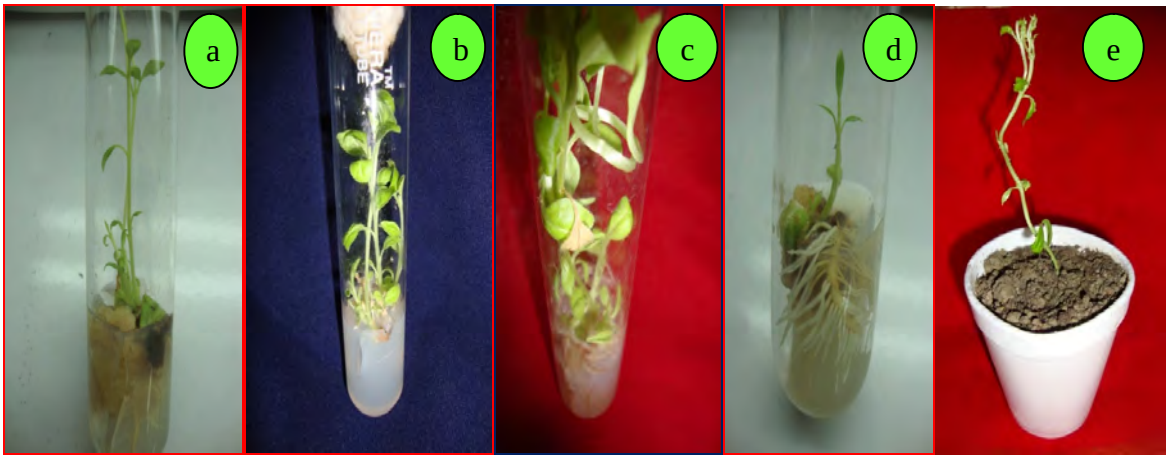
entire explant within 4 weeks. Shoot primordia also developed on the callus, which exposed its organogenic nature. The major portion of the callus was friable and creamy white. Of the various combination sof phytohormone tested, 0.5 mg L^{-1} IAA+ 0.5 mg L^{-1} BAP + 1.0 mg L^{-1} Kn was finest for mean callus induction (32.8%) and days to callus induction (Plate 1a). The combination showed 53% response with respect to the callus induction in shoot apex in 25 days. Mallick *et al*. (2012) also obtained good callus 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}).

Shoot regeneration on callus

Regeneration of adventitious shoots were obtained after 20-25 days of culture from the callus on only two MS medium supplemented with hormonal combination of either 1.0 mg L^{-1} IAA + 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} Kn or 0.5 mg L^{-1} IAA+0.5 mg L^{-1} BAP + 1.0 mg L^{-1} Kn (Table 1). The high level of callus-mediated shoot regeneration was observed when MS medium was supplemented with 1.0 mg L^{-1} IAA + 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} Kn. Low auxin (IAA, 0.5 mg L^{-1}) and high cytokinin (0.5 mg L^{-1} BAP +1.0 mg L^{-1} Kn) gave better mean number of shoots culture^{-1} (6.6) with mean shoot length (3.2 cm) followed by the same basal medium supplemented with auxin (IAA, 0.5 mg L^{-1}) and cytokinin (0.5 mg L^{-1} BAP + 0.5 mg L^{-1} Kn) [Plate 1a]. Better shoot length (2.0 cm) and mean number of shoots culture^{-1} (5.0) were also noticed in this combination. Thus, it reveals that for shoot regeneration a low concentration of auxin and high concentration of cytokinin is necessary. The results are in congruence with the findings of Hans *et al*. (1990) who reported that the medium containing BAP alone (5-25 mg L^{-1}) or in combination with NAA (0.05-1.0 mg L^{-1}) favour better shoot formation from callus. Edson *et al.* (1996) reported that minimal concentrations of cytokinins and auxins in culture media avoid somaclonal variation and efficiently produce true to type plantlets.

Direct shoot regeneration

The initial response of axillary shoot bud formation was observed after 12-18 days of shoot apex explant culturing (Table 1). A progressive increase in axillary shoot bud formation and shoot elongation was observed at lower concentrations of BAP with IAA and Kn, while higher concentrations led to a decrease in shoot induction. Amongst the 7 combinations of shoot-induction media tested, best growth of axillary shoots was observed on MS media supplemented with 0.2 mg L^{-1} IAA + 0.2 mg L^{-1} BAP + 0.2 mg L^{-1} Kn with maximum mean number of shoots culture^{-1} (10.0) and shoot length (4.4 cm) followed by MS medium supplemented with 0.5 mg L^{-1} IAA + 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} Kn. The mean shoot length and mean number of shoots culture^{-1} in this combination were 4.1 and 7.6, respectively (Table 1, Plate 1b). The propagation of plants from



Plates 1*: In vitro* response of shoot apex of *R. serpentine* genotype RS-1; a – Callus-mediated shoot induction; b - Direct shoots proliferation; c - Rooting of regenerated shoots; d - Callus mediated root differentiation; e - Regenerated plantlets in pot

axillary buds proved to be the most generally applicable and reliable method of *in vitro* propagation in *R. serpentine,* as plant regeneration is very difficult from seeds and other sources. The seeds are mostly non-viable due to abortive embryos (Akram and Illahi, 1986). Rani *et al*. (2009) observed high frequency of direct shoot regeneration on media having BAP and Kn from leaf explants in *Chlorophytum borivilianum.* Sivanesen and Murugesan (2005) found only direct shoot regeneration from leaf explant of *Withania somnifera* cultured on MS media supplemented with BAP, Kn and NAA with mean shoot length 1.50 ± 0.28 cm. Harisaranraj *et al.* (2010) reported stunted shoot growth with increase in the concentrations of BAP or Kn which is in accordance with present study.

Root regeneration and acclimatization

Rooting ability of micro-shoots in the present study was satisfactory. *In vitro* regenerated shoots produced profuse rooting 15-20 days after sub-culturing. Highest rooting frequency was obtained in presence of IBA. Of the various hormone combinations tested, IBA (0.5 mg L^{-1}) proved best for higher root induction (84.3%), number of root explants^{-1} (12.3) and root length (6.1). The roots produced were long, pale white and robust with root hairs (Table 2, Plate 1c,d). Our findings are in tune with Rahman *et al.* (2008) who reported that the adventitious shoots were best rooted on half

Table 2: *In vitro* response from regenerated shoots explants of *R. serpentina* for rooting on MS medium supplemented with various phytohormones

Phytohormone (mg L^{-1})			Response (%)	Shoots culture^{-1} (No.) (Mean ± SE)	Shoot length (cm) (Mean ± SE)	Induction of callus (Mean ± SE)	Nature of callus
IBA	IAA	NAA					
0.5	-	-	84.3 ± 2.29	12.33 ± 1.45	6.16 ± 0.29	-	-
1.0	-	-	76.3 ± 3.66	10.33 ± 0.88	4.46 ± 0.52	-	-
1.5	-	-	76.4 ± 0.72	12.33 ± 2.84	3.56 ± 0.23	-	-
2.0	-	-	66.8 ± 1.43	7.66 ± 0.33	2.76 ± 0.14	-	-
1.0	1.0	-	35.7 ± 3.37	3.20 ± 0.12	0.02 ± 0.01	-	-
2.0	2.0	-	22.8 ± 0.80	3.00 ± 0.57	1.00 ± 0.25	-	-
4.0	2.0	-	18.6 ± 0.20	2.33 ± 0.33	1.26 ± 0.37	-	-
4.0	-	2.0	69.5 ± 1.21	7.00 ± 0.57	5.16 ± 0.84	43.06 ± 1.50	F, W
-	-	2.0	64.7 ± 0.37	6.00 ± 0.57	4.93 ± 0.34	44.83 ± 2.33	SF, W
SE(m)±			2.08	1.16	0.40		
$CD_{0.05}$			6.30	3.49	1.20		

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

strength MS medium supplemented with IBA @ 1.0 mg L^{-1}. NAA and IBA reportedly have stimulatory effect on root induction in mulberry (Channd *et al.*, 1995). Susila *et al*. (2013) obtained 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}) + IBA (0.2 mg L^{-1}). The developed plantlets were kept in poly-house and greenhouse for hardening and then transferred to the field. About 70% plants transferred to the field survived and did not show any detectable variation in morphology or growth characteristics as compared to the mother plants (Plate 1e). The use of micro-propagation of *R. serpentina* genotype RS-1 through *in vitro* culture; therefore, offers a mean for large scale production of true-to-type uniform disease-free elite plantlets.

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