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In Vitro Propagation and Conservation of a Medicinal Plant *Rauvolfia serpentina* (L.) Benth. Ex Kurz. From Similipal Biosphere Reserve

Research Article

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Abstract

Rauvolfia serpentina(L.) Benth. ex Kurz is commonly known as Sarpagandha is an important medicinal shrub of family Apocynaceae. This plant species is of great medicinal importance to treat cardiovascular diseases, hypertension, diabetes, malaria, cancer etc. The natural habitat of this species is decreasing due to anthropogenic activities and rapidly eroding natural ecosystem. This plant has comparatively moderate propagation rate in nature. Present investigation is an effort to establish *Rauvolfia serpentina for* direct and indirect organogenesis from nodal and leaf explants. Leaf and shoot explants were cultured on MS media supplemented with different concentrations of NAA, 2, 4-D, BAP and KIN were used either in singly or in combination. Among all the growth hormones 2, 4-D was the best for callus induction (94% in stem and 98% in leaf) and in combination 2, 4-D and BAP (84% in stem and 94% in leaf). Day of callus induction started from13th to 37th day. For direct regeneration from nodal segment, best growth of auxiliary shoot was obtained on MS medium supplemented with 1.5mg/l BAP and 0.5 mg/l NAA concentrations..

Keywords: *Rauvolfia Serpentina;* Callus*; In Vitro* Propagation

Introduction

The typical traditional systems of medicine for thousand years have been in existence have formed from plants. The plant remains to offer mankind with new medicines. At present, many well-established herbal and plant medicine practices (Ayurvedic medicine in India) are popular in many parts of the world. The World Health Organization (WHO) reported that 80% of people in developing world use medicinal plant for their primary health care [1]. The use of herbal medicines is growing in developed countries, presently 25% of the UK population use herbal medicine [2]. About 40% of compounds used in pharmaceutical industry derived directly or indirectly from plants [3] because the chemical synthesis of such compounds is either not possible or economically not visible [4]. Therefore, a large number of medicinal plant species are under threat of extinction because of their overexploitation [5].

Rauvolfia serpentina (L.) Benth ex Kurz is commonly known, as sarpagandha is an important medicinal shrub of family Apocynaceae [6] which grow up to 60m of height. The total numbers of 80 species are included in this genus. It is also known as chandrabhaga, snakeroot plant, chhootachand, Chandrika, Harkaya [7]. Its root contains 0.15% reserpine and rescinnamine group of alkaloids. It also contains a number of bioactive compounds including ajmaline, deserpidine, rescinnnamine and yohimbine. The root of this plant used as medicine for high blood pressure, insomnia, anxiety and other disorders of central epilepsy [8].

The number of *R. Serpentina* species is slowly degrading in India due to over exploitation and random collection for commercial purposes to meet the pharmaceutical industry coupled with limited cultivation [9-11].Another reason for degradation of this plant is poor seed germination. This plant germinates through vegetatively

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by root cutting. The chemical reserpine is an alkaloid first isolated from roots of *R. serpentina* and is used to treat hypertension [12, 13]. Subsequently, other clinical investigators working in India confirmed the effectiveness of *R. serpentina* for that purposes [14, 15]. In short-term study, a significant decrease in systolic as well as diastolic blood pressure of patients was observed [16].The pectic polysaccharide named rauwolfian RS was obtained from the dried callus of *R. serpentina* by extraction with 0.7% aqueous ammonium oxalate and it was found to possess some anti-inflammatory effect [17].Therefore, major steps have been taken to conserve this medicinal plant.

Ex-situ conservation through plant tissue culture is very successful for mass propagation of several plant species. Micro propagation can be considered as an important tool for the production of higher quality based plant-based medicines. Regarding this view there is an urgent need to apply *in vitro* culture methods for micro propagation and conservation of this valuable plant. *In vitro* regeneration of *Rauvolfia* has been reported by many authors [18, 19, 9, 7]. With this insight there is an urgent need to apply in vitro culture methods for micro propagation and conservation of this valuable plant. The present study was undertaken to develop a more efficient protocol for rapid *in vitro* multiplication of *R. serpentina*.

Materials and Methods

Collection and sterilization of explants

R.serpentina was collected from Similipal Biosphere Reserve and was planted in departmental garden of Dept. of Botany, MSCBU, Takatpur, Baripada. Young stem (nodal) and leaves of *R. serpentina* were taken as explants in the present study. The explants were thoroughly washed with running tap water followed by double distilled water and 70% ethanol for 30 seconds. These were also treated with labolene solution for 5 minutes under Laminar airflow. Subsequently the stems and leaves were finally sterilized with 1% of sodium hypochlorite solution (NaOCl) for 10 minutes and then washed with sterile distilled water for 4-5 times. Stems and leaves were then dried using sterile tissue paper and excised into segment and inoculated onto prepared medium.

Preparation and sterilization of media

For *in vitro* culture of *R. Serpentina* MS medium [20] was taken. To prepare 1 litre medium requisite amount of sucrose (30 g/l) and agar (8g/l) were added. Various growth regulators like auxins or cytokinins were also added according to required amount. The pH of the medium was adjusted to 5.6 to 5.8 and the media were autoclaved at 15 Ibs/inch^2 for 20 minutes at 121°C for proper sterilization.

Callusing

For callus induction, juvenile stem (nodal) and leaf cut about 5 mm in length were aseptically prepared and were implanted vertically on MS medium prepared with specific concentrations of hormones. Culture of stem and leaf explants were initially incubated under darkness in a culture chamber at 25°C for callus induction. Subsequently, explants were incubated under a 16/8-h (light/ dark) photoperiod with cool-white fluorescent lighting at an intensity of 60 µE·m−2·s−1 intensity at a constant temperature of 25°C ± 2°C.

Direct regeneration of Shoot

For direct shoot regeneration, the nodal segment was cut into small pieces and each piece of nodal segment was transferred to MS media having growth hormones in similar composition and concentration as for shoot regeneration and light treatments were same as for callus induction. After 3-4 weeks, direct shoot regeneration occurs from nodal segment. After 5-6 weeks of old shoots were cut into 3.5 cm in length and cultured on MS media having same growth hormones in similar composition, concentration and incubation as for shooting and after 75 days complete plantlets were formed.

Results

Effects of different concentrations of auxin and cytokinin singly on callus induction

MS media supplemented with different concentrations of (Figure 1) 4-Dichlorophenoxyacetic acid (Figure 1), (Figure1-D) showed stimulatory effects on callus induction. Maximum callusing response (94% in stem and 98% in leaf) was noted at 2.5mg/l. At 10mg/l no callusing or growth was observed.

MS media supplemented with different concentrations of 1-Naphthaleneacetic acid (NAA) showed stimulatory effects on callus induction. Maximum callusing response (72% in stem and 78% in leaf) was recorded at 2 mg/l of NAA. At 0.5mg/l the callusing response was recorded less and it increased up to 2mg/l. At 2.5mg/l onward callusing response was reduced and found minimum at 5mg/l. At 10mg/l no callusing or growth was observed. It was observed that the higher concentration of NAA in media had an inhibitory effect on callus proliferation.

MS media supplemented with different concentrations of 6-Benzyl aminopurine (BAP) showed stimulatory effects on callus induction. Maximum callusing response (66% in stem and 70% in leaf) was noted at 2.5mg/l of BAP. Lower concentrations of BAP (0.5mg/l to 1.5mg/l) were unable to induce callusing and higher concentrations of BAP (10 mg/l) in media had an inhibitory effect on callus induction.

But MS medium supplemented with concentrations of 0.5 mg/l to 10 mg/l of Kinetin (KIN) callus formation was not observed on stem and leaf explants.

Effects of different concentrations and combinations of growth hormones on leaf and stem callus induction

MS media supplemented with different concentrations of (Figure 1), (Figure1-D)-Dichlorophenoxyacetic acid (Figure 1), (Figure1-D) and 6-benzylaminopurine (BAP) showed stimulatory effects on callus induction. Maximum callusing response (84% in stem and 94% in leaf) was recorded at 1mg/l of BAP and 2mg/l of (Figure 1), (Figure1-D). At 3mg/l of BAP and 1 mg/l of (Figure 1), (Figure1-D) swelling of callus was observed. At 5mg/l to 10mg/l of BAP and (Figure 1), (Figure1-D)no callusing or growth was observed.

MS media supplemented with different concentrations of 1-Naphthaleneacetic acid (NAA) and 6-Benzylaminopurine (BAP) showed stimulatory effects on callus induction. Maximum callusing response (76% in stem and 82% in leaf) was recorded at 0.5 mg/l of

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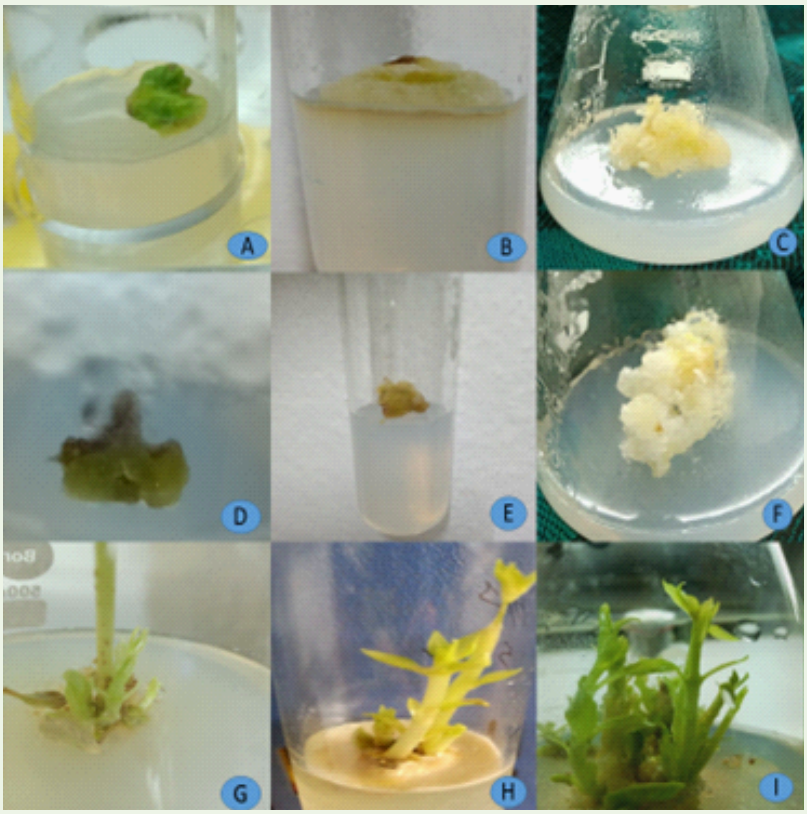

Figure 1: A-I: Micropropagation of Rauvolfia Serpentina; (A-C): Callus Formation from Leaf Explants; (D-F): Callus Formation from Nodal Explants; (G-I): Multiple Shoot Formation from Nodal Explants.

BAP and 1 mg/l of NAA. At 2.5 mg/l to 10 mg/l of BAP and NAA no callusing or growth was observed.

MS media supplemented with different concentrations of 1-naphthaleneacetic acid (NAA) and KIN showed stimulatory effects on callus induction. Maximum callusing response (64% in stem and 72% in leaf) was recorded at KIN 1 mg/l and NAA 1.5 mg/l. At 2.5 mg/l to 10mg/l of KIN and NAA no callusing or growth was observed.

Direct shoot regeneration from nodal segment

The plant is vegetatively propagated by root cutting because of seed are mostly non-viable due to abortive embryos and low germination percentage. The *in vitro* multiplication of *R. serpentina* shoots through nodal segment is the most commercially viable means of micro propagation. It is also useful for increasing number of shoots, which originally differentiated *in vitro*. The present study deals with the *in vitro* propagation for direct regeneration from nodal segments *R.serpentina*. Best growth of axillary shoots were obtained from MS media supplemented with 1.5 mg/l BAP and 0.5 mg/l NAA concentrations were applied to induce direct regeneration followed by BAP 1 mg/l and NAA 1 mg/l in nodal explants.

Discussion

In the present work, two explants leaf and nodal stem were used in which leaf explants were found best for callus induction than stem, which is in accordance with the earlier findings [21] . *In vitro* regeneration of *R. serpentina* has been reported by many authors [22-26]. For the preparation of media standard procedure was followed [27]. MS media without any growth hormone was unable to induce callus [27]. The auxins facilitate cell elongation and root initiation while the cytokinins induce cell division and differentiation. Among all the growth hormones, (Figure 1), (Figure1-D) was the best for callus induction in leaf explants (Table-1) (Figure-1C). Present results are also in accordance with the result reported by [23] Mitra and Kaul in 1964. (Figure 1), (Figure1-D)is an elective herbicide with auxin activity. The herbicide is especially designed to control broad leaf weeds (dicotyledons) in cereal crop fields. It is generally accepted that (Figure 1), (Figure1-D)is an auxin-like herbicide, because at low concentration it has growth promoting properties. The first herbicide reported to improve growth and yield of crops at sub toxic level was (Figure 1), (Figure1-D) [28]. (Figure1), (Figure1-D)is usually used with cytokinins for callus induction. The induction of callogenesis was also conformed in *Kalanchoe blossfeldiana* pollen and *Digitalis lanata*Ehrh [29, 30]. (Figure2), (Figure1-D) is an auxin and plays a primary role in cell elongation and root initiation [31]. The response of explants in this study might be due to the auxin- cytokinin balance derived from exogenous auxin (Figure1), (Figure1-D) and endogenous auxin cytokinin in plant cells.

The cytokinins and auxins are important in in-vitro culture

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Table 1: Effectof plant growth hormones on leaf explants for callus induction

Phytohormone conc. (mg/l)				Leaf percentage of callus response	Days of callus induction	Degree of callusing
2,4-D	BAP	NAA	KIN			
0.5				-	-	-
1.0				-	-	-
1.5				-	-	-
2.0				82±3.0	20±1.7	+++
2.5				98±1.1	19±1.2	+++
3.0				22±2.4	29±1.7	+
4.0				20±1.3	27±2.6	+
5.0				16±2.4	31±2.9	+
	0.5			-	-	-
	1.0			-	-	-
	1.5			-	-	-
	2.0			62±3.0	20±1.1	++
	2.5			70±4.1	18±1.5	++
	3.0			34±2.0	24±2.3	+
	4.0			26±2.3	29±1.1	+
	5.0			16±1.1	31±1.0	+
2.0	0.5			48±3.0	25±1.7	+
2.0	1.0			68±2.3	21±1.5	++
1.5	1.0			18±5.0	33±2.0	+
2.0	1.5			94±2.3	19±2.5	+++
2.5	1.5			18±3.0	33±1.1	+
1.0	1.5			46±3.0	27±2.0	+
2.0	2.5			32±2.3	29±2.6	+
1.0	2.5			22±1.1	33±1.1	+
		0.5		24±1.1	28±2.0	+
		1.0		62±0.6	20±1.5	++
		1.5		68±2.3	19±1.7	++
		2.0		78±2.0	19±3.0	+++
		2.5		32±3.0	27±2.0	+
		3.0		24±4.0	28±3.4	+
		4.0		18±2.0	30±1.5	+
		5.0		14±1.1	33±1.1	+
	0.1	2.0		52±2.3	26±2.3	+
	0.5	1.0		82±3.0	21±1.7	+++
	1.0	1.5		18±1.1	33±1.1	+
	1.0	2.0		64±2.3	20±1.5	++
	1.5	1.5		24±2.0	35±1.1	+
		0.5	0.5	28±3.0	29±0.5	+
		1.0	1.0	60±1.1	20±1.1	++
		1.5	1.5	72±2.3	19±1.7	++
		2.0	2.0	56±5.0	21±1.1	++
		1.5	2.5	24±3.0	30±1.5	+
		2.0	3.0	42±2.3	24±2.3	+

as the later are concerned with root formation, and the former are mainly required for shoot formation and growth of buds [32]. These growth regulators are required in combination as it is always the manipulation and variation in auxins and cytokinins level that can successfully change the growth behaviour of plant cultures [34]. Cytokinins such as BAP and kinetin are known to reduce the apical meristem dominance and induce both auxiliary and adventitious shoot formation [35]. The application of higher concentration of BAP inhibits elongation of adventitious meristems and the conversion into complete plant. Auxins and other growth regulators play important roles in growth and differentiation of cultured cell and tissue [36]. In our present study we found that BAP alone is less effective in callus formation than BAP in combination with auxins like 2,4-D and NAA (Table-1), (Figure-1F). Similar results are reported by [37] Roja and Heble in 1996 where MS media supplemented with 2, 4-D and BAP was found best for callus induction.Some workers have reported that BAPin combination with other auxins like IAA and IBAshows positive results in callus formation of *R. Serpentina* [9, 38] which is different from our results.

NAA comes under auxin family plays an important role in rooting agent. NAA is too toxic at higher concentration to plants but at lower concentration, it shows growth and development of plant tissue [39]. In our present study, NAA in combination with cytokinin like BAP shows better results in callus regeneration than alone (Table-1), (Table-2). In the present work phytohormone named Kinetin (KIN) alone could not induce callus [40]. In further experiments Kinetin was supplemented to the MS media in combination with auxins (2, 4-D and NAA). It was observed that KIN had enhanced callus growth in presence of auxins. Day of callus induction started from 17th to 37th day [41]. This variation observed in the present investigation may be attributed due to the difference in culture conditions and the age of explants.

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Table 2: Effectof plant growth hormones on stem nodal explants for callus induction

Phytohormone conc. (mg/l)				Stem percentage of callus response	Days of callus induction	Degree of callusing
2,4-D	BAP	NAA	KIN			
0.5				-	-	-
1.0				-	-	-
1.5				-	-	-
2.0				78±2.4	21±1.5	+++
2.5				94±3.0	20±2.1	+++
3.0				24±1.1	31±1.5	+
4.0				18±1.7	33±1.2	+
5.0				14±2.3	35±0.6	+
	0.5			-	-	-
	1.0			-	-	-
	1.5			-	-	-
	2.0			58±5.2	21±1.1	++
	2.5			66±4.1	20±2.0	++
	3.0			30±5.0	27±1.5	+
	4.0			22±3.4	31±1.5	+
	5.0			14±2.3	34±0.5	+
2.0	0.5			66±2.3	23±1.5	++
2.0	1.0			72±4.1	20±2.6	++
1.5	1.0			20±3.0	31±1.7	+
2.0	1.5			84±3.4	19±2.0	+++
2.5	1.5			22±1.1	31±2.0	+
1.0	1.5			42±3.4	25±1.5	+
2.0	2.5			38±2.3	26±2.3	+
1.0	2.5			24±1.1	30±1.7	+
		0.5		22±1.7	29±1.1	+
		1.0		58±3.0	21±0.5	++
		1.5		64±2.0	21±1.6	++
		2.0		72±2.3	20±2.0	+++
		2.5		28±4.1	28±1.7	+
		3.0		20±1.1	31±1.5	+
		4.0		16±2.4	34±1.1	+
		5.0		12±1.1	36±1.1	+
	0.1	2.0		62±2.3	24±1.1	++
	0.5	1.0		76±3.4	21±1.5	+++
	1.0	1.5		22±1.1	32±1.0	+
	1.0	2.0		58±2.3	22±2.0	++
	1.5	1.5		18±1.1	33±1.7	+
		0.5	0.5	24±2.3	31±1.1	+
		1.0	1.0	56±3.0	24±2.0	++
		1.5	1.5	64±4.1	20±1.5	++
		2.0	2.0	52±3.0	23±1.7	++
		1.5	2.5	20±3.0	31±0.5	+
		2.0	3.0	38±2.3	26±3.0	+

Table 3: Direct shoot regeneration from stem nodal explants on MS medium under the influence ofdifferent concentrations of phytohormones.

Phytohormone concentration (mg/l)		% of shoot formation	Degree of shooting	Days of shoot regeneration	Mean length of shoot
BAP	NAA				
0.5	0.5	26±2.3	+	32±1.6	2.5±0.4
1	0.5	40±2.0	+	30±1.2	3.2±0.2
1	1	80±1.1	++	28±2.3	4.2±0.3
1.5	0.5	94±2.4	+++	26±1.1	7.1±1.2
2	1	44±1.2	+	30±1.5	3.8±0.1

The *in vitro* multiplication of *Rauvolfia serpentina* shoots through nodal segment is the most commercially viable means of micro-propagation. It is also useful for increasing number of shoots, which originally differentiated *in vitro*. Best growth of axillary shoots was obtained on MS supplemented with concentration and combination of phytohormones containing 1.0 mg/l BAP and 0.1 mg/l NAA (Table-3), (Figure-I). Thus, the propagation of plants from nodal segments has proved to be the most generally applicable and reliable method of *in vitro* propagation in *Rauvolfia 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 [42,43].

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The present work describes a reproducible and efficient protocol for propagation of *R. serpentina* as an important medicinal plant species from leaf and stem explants. Propagation from nodal explants through direct organogenesis removes the need for an intervening callus phase and avoids the use of mercuric chloride, thus assuring the species effective establishment and multiplication irrespective of seasonal constraints. This micro propagation technique could support the conservation of this valuable plant species to protect it from indiscriminate exploitation. It could work as useful tool to increase biomass and yield of pharmaceutically important alkaloids and photochemical accumulated in *Rauvolfia serpentina*.

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