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An Efficient *in vitro* Clonal Propagation and Estimation of Reserpine Content in Different Plant Parts of *Rauwolfia serpentina* L.

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Abstract: A highly efficient protocol for *in vitro* propagation of *Rauwolfia serpentina* L. was developed that consisted of callus induction, multiple shoot regeneration and rooting stages. Effect of different plant growth regulators, exclusively or in combination on callus induction, multiple shoot formation and root induction, were observed. Maximum callusing (93.65%) were obtained in leaf and stem explants inoculated in Murashige & Skoog's (MS) medium containing 2,4 - dichlorophenoxy acetic acid (2.0 mg l^{-1}) plus 6-benzyl amino purine (1.0 mg l^{-1}). Maximum shoots (25.4) per culture were obtained from the callus inoculated in shooting medium containing BAP (2.0 mg l^{-1}) plus NAA (0.5 mg l^{-1}). The rooting was induced in *in vitro* regenerated shoots in half strength MS medium containing IBA (0.2 mg l^{-1}) plus NAA (0.2 mg l^{-1}) and 100% rooting was obtained. The *in vitro* regenerated plantlets were transferred to the glasshouse with 75% success. The reserpine content was measured in the different parts of *Rauwolfia serpentina* plant including, leaf, stem, flower and root. The 90% of the total reserpine content was produced from the root, with the stem and leaf containing 10%, it proves the tissue specificity of the reserpine production.

Key words: Alkaloids · Callusing · Multiple shoots · Plant growth regulators · *Rauwolfia serpentina*

INTRODUCTION

Rauwolfia serpentina L. Benth Kurz commonly known as sarpgandha, is a small, woody, perennial medicinal shrub which belongs to family Apocynaceae. The root of *R. serpentina* is a rich source of indole alkaloids *viz.* reserpine, recinnamine, serpentine, ajamaline, ajmalicine, etc. According to ayurveda its root and whole plant is used for the treatment of cardiovascular disorder, snake bite, rheumatism, hypertension, insanity, epilepsy, eczema and leaves are used in removal of opacities of the cornea [1, 2]. After reports of therapeutic properties, natural reserves of *R. serpentina* L. are declining due to overexploitation by the local and tribal people. The International Union for the Conservation of Nature and Natural Resources (IUCN) has assigned an endangered status to *R. serpentina* [3, 4].

There are several earlier attempts, to develop *in vitro* propagation protocol, for rapid and large scale multiplication of *R. serpentina* via callus, shoot tips, axillary buds and nodal segment explants in MS medium containing different concentration of BAP & NAA [5-15].

In vitro propagation offers not only a means for mass multiplication of existing germplasm stocks, but also for the conservation of important elite and RET (rare, endangered & threatened) species which are facing the danger of extinction.

Pharmaceutical importance and the natural status of the plant motivated us to develop a protocol for the efficient multiplication and conservation of elite germplasm of *Rauwolfia serpentina* and further to investigate the distribution pattern of reserpine content in different parts of the plant.

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MATERIALS AND METHODS

Young leaf and stem explants were collected from one year old field grown plants of *Rauwolfia serpentina.* The explants were washed properly in running tap water for 30 minutes followed by washing with 1.0% solution of Twene-20 for 25 minutes. Explants were rinsed properly until the detergent was completely washed out. To prevent fungal contamination, explants were treated with 6-10% bavistin. Finally, the explants were treated with 70% ethanol for one minute followed by 0.1% $HgCl_2$ for 1.0 minute. After each treatment, explants were rinsed three times with sterilized double distilled water. All the operations were performed in the laminar airflow.

Sterilized leaves (0.5 cm^2) and young stem (1.0 cm long) explants were carefully inoculated in MS medium supplemented with various concentrations of growth regulators, including (2,4-D, NAA, BAP, kinetin etc.) exclusively or in combination. The pH of the medium was adjusted to 5.8±1.0. Cultures were grown at 25°C temperature and 16h/8h light period for callus induction. One month old calli were cut into small pieces and sub cultured in the same medium for further proliferation.

To observe the optimal concentration for multiple shoot induction the calli were transferred to MS medium containing BAP (0.5 - 2.5 mg l^{-1}) kinetin (0.5 - 2.5 mg l^{-1}) and NAA (0.1 - 0.5 mg l^{-1}) separately or in different combinations.

The *in vitro* regenerated multiple shoots were transferred to half-strength MS medium containing auxins, *viz.* NAA, IBA and IAA, at a different concentration and combination for the root induction. The plantlets with well-developed roots were taken out from the culture tubes and transferred to the glasshouse. The plantlets were put into individual pots containing compost and then put into a container. The container was covered with polyethylene bags that were opened at increasing intervals to help the plantlets to the lower humidity of the glasshouse.

Extraction and Estimation of Reserpine: Whole plants were removed from the flasks and freeze-dried for 24 h. For each sample, 100 mg powder was extracted using 5 ml of methanol for 20 minutes. The extract was evaporated to dryness in the soxhlet evaporator. The crude extract obtained was dissolved in 100 ml of 0.01 M HCl. The pH of filtered solutions were adjusted to 6.0 with 0.01 M NaOH. The crude reserpine obtained was used for HPLC analysis [16].

Quantitative determinations of reserpine were accomplished by HPLC using reversed phase C_{18} column packed with spherisorb S_5 ODS-2. A stainless steel columns (Luna 5µ, C_{18} (2), 250×4.60 mm- Make Phenomenax, USA) was used in the study. The samples were eluted in a mobile phase of methanol: acetonitrile (60:40 v/v) at a flow rate of 1.0 ml/min. The compounds were detected at a wave-length of 254 nm. The reserpine standard was purchased from Hi-media Laboratories. All experiments were performed in triplicates and the results expressed as mean values.

RESULTS

Callus Induction: The leaf and stem explants inoculated in basal MS_0 medium did not induce any calli. Supplementing the medium with various concentrations of auxin and cytokinin induced callusing in both the explants. Both leaves and stem explants produced calli in MS medium supplemented with 2,4-D (1.5-2.0 mg l^{-1}) and BAP (0.2-1.5 mg l^{-1}). The best response was obtained in MS medium, supplemented with 2,4-D (2.0 mg l^{-1}) and BAP (1.0 mg l^{-1}) and 93.65% callusing was reported (Table1).

Table 1: Callus induction in leaf and stem explants of *R. serpentina* L. in MS medium supplemented with different concentration and combination of 2, 4-D, NAA, BAP and kinetin.

	% Callus induction	
Medium composition	Leaf	Stem
MS_0	-	-
2,4-D + BAP		
1.5 + 1.0	20.18	19.13
1.5 + 1.5	23.21	20.14
2.0 + 0.2	65.00	46.98
2.0 + 0.5	72.35	68.56
2.0 + 1.0	93.65	85.37
2.0 + 1.5	42.02	45.00
NAA + Kinetin		
1.5 + 1.0	18.12	16.12
1.5 + 1.5	21.51	21.00
2.0 + 0.2	25.12	24.69
2.0 + 0.5	52.32	48.45
2.0 + 1.0	72.68	62.99
2.0 + 1.5	19.17	32.14
2.5 + 0.2	-	-
2.5 + 0.5	17.23	23.14
2.5 + 1.0	31.38	36.37
2.5 + 1.5	11.31	19.36

* Mean of three replicates of leaf & stem derived callus

Fig. 1: Repersentative photos of the sequence of events and the stages of *in vitro* plantlet development and hardening of *Rauwolfia serpentina* L.:

a) 30 days old callus regenerated in Basal MS medium supplemented with 2,4-D (2.0 mg l^{-1}) and BAP (1.0 mg l^{-1}) from leaf explants.
b) Callus transferred to shoot induction medium containing BAP (2.0 mg l^{-1}) & NAA (0.5 mg l^{-1}).
c) One month old fully developed multiple shoots
d) Culture showing well rooted plants in half strength MS medium containing NAA (0.2 mg l^{-1}) and IBA (0.2 mg l^{-1}). e) & f) Hardening of *in vitro* developed shoots

Proliferation of Callus: One-month-old callus, obtained from leaves and stem explants, was sub cultured in the same medium for its proliferation. Green, friable callus was obtained after 3-4 weeks of subculture. (Fig.1-a). Callus obtained from the leaf explants was loose in texture while that from the stem explants was tough and compact.

Multiple Shoot Induction: The callus derived from leaves and stem explants failed to produce multiple shoots in basal MS medium. The medium supplementing with BAP (1.5 - 2.0 mg l^{-1}) produce multiple shoots and the shoot induction percentage ranges between 22.87 - 56%. However, at higher concentration of BAP the number

Table 2: Multiple shoot induction in the callus of *R. serpentina* L. in MS medium supplemented with different concentration and combination of BAP, kinetin, NAA and IBA

Growth Regulators (mg/l)	% of Shoot formation	Average no of shoots per culture*	Average length of shoots (cm)*
MS_0	-	-	-
BAP			
0.5	-	-	-
1.0	-	-	-
1.5	22.87	2.8	1.8
2.0	56.76	4.3	2.4
2.5	43.23	3.3	1.9
BAP+ NAA			
0.5 + 0.1	-	-	-
1.0 + 0.2	-	-	-
1.0 + 0.3	16.00	2.3	2.1
1.0 + 0.4	22.78	2.8	2.5
1.0 + 0.5	26.86	2.8	2.9
1.0 + 0.6	31.89	3.1	3.0
1.5 + 0.1	19.93	3.3	2.3
1.5 + 0.2	24.56	3.5	2.6
1.5 + 0.3	29.45	3.8	3.1
1.5 + 0.4	35.12	4.0	3.3
1.5 + 0.5	44.76	4.5	3.1
1.5 + 0.6	48.23	4.8	3.2
2.0 + 0.1	22.87	3.8	3.2
2.0 + 0.2	29.00	4.6	3.6
2.0 + 0.3	38.89	5.6	4.0
2.0 + 0.4	78.98	6.2	4.5
2.0 + 0.5	95.34	25.4	5.1
2.0 + 0.6	65.12	5.8	3.7
BAP + IBA			
1.0 + 0.1	-	-	-
1.0 + 0.2	18.67	1.8	2.1
1.0 + 0.3	24.54	2.3	2.4
1.0 + 0.4	28.76	2.6	2.6
1.0 + 0.5	33.43	3.0	2.4
1.5 + 0.1	19.22	3.2	2.3
1.5 + 0.2	23.12	3.4	2.6
1.5 + 0.3	26.31	3.6	3.1
1.5 + 0.4	31.54	3.9	3.1
1.5 + 0.5	41.35	4.3	3.6
2.0 + 0.1	21.43	3.3	3.1
2.0 + 0.2	25.00	4.1	3.4
2.0 + 0.3	36.59	5.3	3.8
2.0 + 0.4	55.78	5.6	4.3
2.0 + 0.5	75.44	16.2	4.7

Table 2: Continue

Growth Regulators (mg/l)	% of Shoot formation	Average no of shoots per culture*	Average length of shoots (cm)*
Kinetin + NAA			
1.0 + 0.1	-		
1.0 + 0.2	12.00	1.7	2.0
1.0 + 0.3	17.65	2.4	2.4
1.0 + 0.4	23.54	2.5	2.3
1.0 + 0.5	29.59	3.1	2.4
1.5 + 0.1	16.63	3.3	2.3
1.5 + 0.2	22.66	3.3	2.5
1.5 + 0.3	26.44	3.5	3.2
1.5 + 0.4	31.22	3.7	3.1
1.5 + 0.5	43.56	4.4	3.4
2.0 + 0.1	20.87	3.1	3.5
2.0 + 0.2	26.80	3.9	3.4
2.0 + 0.3	34.87	4.3	3.7
2.0 + 0.4	55.48	5.8	4.2
2.0 + 0.5	72.34	14.6	4.5

* Mean of three replicates of *in vitro* regenerated shoots

Table 3: Root induction in *in vitro* regenerated shoots of *R. serpentina* L. in half strength MS medium supplemented with different concentration of IAA, NAA and IBA exclusively or in combination

Growth Regulators (mg/l)	% rooting per shoot*	Average length of root (cm)*
½ MS_0	-	-
IAA		
0.1	20	1.2
0.2	26	1.8
0.3	20	1.3
0.4	17	1.2
0.5	10	1.0
NAA		
0.1	13	1.8
0.2	36	2.2
0.3	17	2.0
0.4	13	1.9
0.5	12	1.5
IBA		
0.1	35	2.4
0.2	48	2.9
0.3	33	2.1
0.4	24	2.4
0.5	12	1.7
NAA + IBA		
0.1 + 0.1	56	2.9
0.2 + 0.2	99.95	4.5
0.3 + 0.3	73	3.1
0.4 + 0.4	51	2.7
0.5 + 0.5	25	2.4
IAA + IBA		
0.1 + 0.1	43	2.3
0.2 + 0.2	66.44	2.9
0.3 + 0.3	39	2.1
0.4 + 0.4	28	1.8
0.5 + 0.5	17	1.1

* Mean of three replicates of *in vitro* regenerated roots

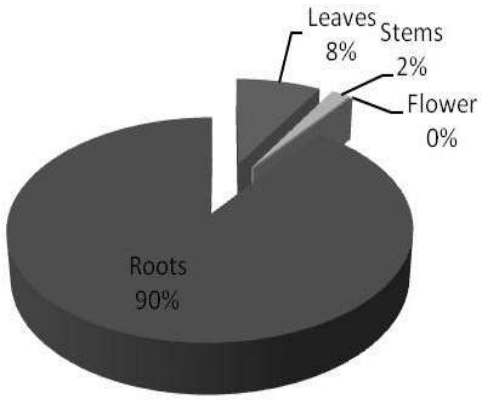

Fig. 2: Distribution percentage of reserpine in each tissue of the *R. serpentina* whole plant cultured after two months in the culture room under 16/8 h light period.

of shoots per culture declined gradually. Table 2 shows that the medium containing BAP (2.0 mg l^{-1}) plus NAA (0.5 mg l^{-1}), BAP (2.0 mg l^{-1}) plus IBA (0.5 mg l^{-1}) and Kinetin (2.0 mg l^{-1}) plus NAA (0.5 mg l^{-1}) enhanced the multiple shoot induction at rate of 95.34%, 75.44% and 72.34%, respectively. Medium containing BAP (2.0 mg l^{-1}) plus NAA (0.5 mg l^{-1}) was observed as the most effective combination for multiple shoot induction and 25.4 shoots per culture were obtained. (Fig.1- b & c).

Rooting and Hardening: The *in vitro* regenerated multiple shoots were transferred to the rooting medium (half-strength MS medium) supplemented with different auxins *viz.* IAA, NAA, IBA. Rooting was obtained in the presence of all the three auxins. However, when the medium was supplemented with NAA (0.2 mg l^{-1}) plus IBA (0.2 mg l^{-1}) 100% rooting was achieved (Fig. 1-d, Table 3).

The plantlets with well-developed roots were transferred to pots in the glasshouse and were acclimatized to open environment with 75% success (Fig.1-e & f).

Reserpine Content in Each Tissue of Whole Plant Culture: To elucidate tissue specificity of reserpine production in *R. serpentina,* we analyzed reserpine content in each tissue of the whole plants, which were cultured for 8 weeks in culture room containing 0.5 l of MS liquid medium. As shown in Figure 2, 90% of total reserpine content was obtained from the root, with the stem and leaf containing 10%. There was no detectable reserpine in the flowers.

DISCUSSION

In the present study, MS medium supplemented with 2,4-D (2.0 mg l^{-1}) and BAP (1.0 mg l^{-1}) was found to be highly conducive for callus induction from both leaves and stem explants of *R. serpentina*. On the other hand, NAA (1.5-2.5 mg l^{-1}) and Kinetin (1.0-1.5 mg l^{-1}) had a negative effect on callus induction. In our study 2, 4-D and BAP were reported to be the most effective for callusing in *R. serpentina,* which is in accordance with the earlier findings [9, 12, 14, 17]. Sehrawat *et al*. [11] stated that callusing was started after 22 days of inoculation, while in our findings callus induction in the leaves and stem explants were initiated within one week of inoculation. The variation observed in the present investigation may be attributed due to the difference in culture conditions and the age of explants.

The MS medium, supplemented with BAP (2.0 mg l^{-1}) plus NAA (0.5 mg l^{-1}), was reported as a most suitable combination for the multiple shoot induction and 25.4 shoots per culture was obtained. In our results, the number of shoots obtained, were significantly higher than the earlier reports [11-15].

In vitro regenerated shoots produced profuse rooting in half strength MS medium containing different concentration of auxins *viz.* NAA, IAA and IBA either alone or in combination. Basal MS medium devoid of auxin could not induce rooting. The plantlets with well-developed roots were obtained after 3-4 weeks of inoculation. The number and percentage of roots per shoot was noticeably influenced by the combination, concentration and type of auxin. IBA was found to be the most effective as compared to NAA and IAA. The combination NAA (0.2 mg l^{-1}) plus IBA (0.2 mg l^{-1}) was found to be the most favourable for proper rooting. In the present investigation 100% rooting were observed which is consistent with the earlier findings on rooting of *in vitro* proliferated shoots of *R. serpentina* [5, 12, 14, 18], but the significant differences were observed in terms of number of roots per culture and length of the roots.

The biosynthesis of majority of secondary metabolites shows tissue specificity. For example, pyrrolizidine alkaloids [19] and avenacins [20] are produced in the roots and similarly asiaticoside triterpene is produced by the leaves [21]. Our findings suggest that reserpine production, which mainly occurs in the roots of *R. serpentina,* is tissue specific.

In the present investigation, the multiple shoot induction percentage, in both leaf and stem derived callus, was higher (95.34%) and also the number of shoots per culture was higher than earlier findings and it was also found that half strength MS medium supplemented with IBA (0.2 mg l^{-1}) and NAA (0.2 mg l^{-1}) induce profuse rooting. The production of large no of multiple shoots and root biomass is one of the significance of this protocol, to obtain medicinally important alkaloids and simultaneously to conserve the natural reserves of *R. serpentina.*

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