Research Article **CODEN: IJPRNK** **ISSN: 2277-8713**
T. Murab, IJPRBS, 2015; Volume 4(3): 398-408 **IJPRBS**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL RESEARCH AND BIO-SCIENCE

IN VITRO PROPAGATION OF *RAUWOLFIA SERPENTINA* (LINN.) BENTH: AN ATTEMPT TO SAVE AN ENDANGERED MEDICINAL PLANT

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Accepted Date: 21/06/2015; Published Date: 27/06/2015

Abstract: *Rauwolfia serpentina* commonly known as Indian Snakeroot or *Sarpagandha* is an important Indian medicinal plant used as antihypertensive agent. In the present study an efficient protocol was established using in vitro micropropagation method for development of plantlet from nodal explants with multiple shooting followed by rooting using various combinations of growth hormones. This protocol will aid in the conservation of endangered medicinal plant i.e., *Rauwolfia serpentine* thereby helping in mass propagation of plant. The nodal explants were cultured on MS medium containing varying concentration of Kinetin (K) in combination with Indole-3- acetic acid (IAA) produced multiple shoots. Maximum multiple shoots induction from axiallary bud occurred in MS media containing 2.5 mg/l K + 0.4 mg/l IAA while root induction was best in MS media containing 0.6 mg/l K + 2.0 mg/l IAA mg/l and callus was induced in MS media containing 0.8 mg/l K +1.8 mg/l 2,4-D.

Keywords: *Rauwolfia serpentine*, endangered, nodal explants, Indole-3- acetic acid, Kinetin.

PAPER-QR CODE

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Access Online On:

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How to Cite This Article:

T. Murab, IJPRBS, 2015; Volume 4(3): 398-408

INTRODUCTION

Rauwolfia serpentina is said to appear in Sanskrit as an Ayurvedic medicine named Sarpgandha and Chandra. Sarpgandha, snakes smell or repellant, refers to the use as an antidote for Snake-bite[1]. *Rauwolfia serpentina* (L.) Benth. Ex Kurz. (Apocyaneae) a woody perennial shrub, is an endangered medicinal plant found in Bangladesh, China, Indonesia, India, Malaysia, Nepal, Pakistan, Sri Lanka and Viet Nam[2],[3] India which holds almost a world monopoly has been threatened with the depletion of wild resource of the plant with the increasing demand. Despite their wide geographical distribution and edaphic tolerance, *Rauwolfia* species have not lent themselves to easy cultivation due to various factors which influence their propagation, growth and development and also their alkaloid content. At present all supplies of *Rauwolfia serpentina* roots are furnished by wild plants. Since supplies from wild sources are limited, it may not be possible to maintain a sustained and steady supply at the present rate of exploitation[4]. The ecological plunder as well as difficulties associated with seed germination in this biotechnologically significant plant has led *Rauwolfia serpentina* to figure in the Red Data Book[5]. Propagation by direct sowing of seeds in the field has not been found successful. Sun-dried and stored seeds generally gave a low rate of germination and seeds stored more than 7-8 months practically did not germinate. The germination percentage of seed is very poor and variable (25-50 %) and is often as low as 10 percent. Propagation by means of seeds might prove ultimately even unwise, since variation in alkaloid yield is apparently genetically controlled and might get reduced in successive progenies through adverse gene recombination[6].

The objective of the present study is to describe an efficient methods of direct plant regeneration from *in vitro* culture nodal explants to induce multiple shooting and rooting, optimization of various media using varying concentrations of hormones for regeneration protocols for shoot elongation and multiple shooting and root generation by standardizing media to develop a reproducible and efficient regeneration of plantlets for the conservation of our this endangered medicinal plant for commercial exploitation.

2. MATERIALS AND METHODS

2.1. Plant Materials and Culture Establishment

The plant of *Rauwolfia serpentina* was taken from the garden of Career college, Bhopal, India. The explants were washed with running tap water for 20 min followed by cutting of 1.5 cm long nodal explants and then soaking the nodal explants in 5 - 10 drops/l of Tween-20 for 20 min followed by washing the explants with distilled water 4- 5 times till the froth cleared. These explants were immersed in 1% (v/v) Savlon solution and were transferred in the laminar airflow cabinet where further surface sterilization of explants was done using varied concentration of

mercuric chloride and 1% ($HgCl_2$) treatment for 2 minutes was found optimal (Table 1). The explants were rinsed 5-6 times with sterile distilled water. After surface sterilization of the explants under laminar air flow cabinet the explants were inoculated and cultured in the MS[7] basal medium containing 3.0% sucrose (w/v), 1% agar.

2.2 Tissue culture Medium and plant growth regulators:

Selection of responsive combination of growth hormones preliminary experiments were performed using two auxins namely indole- 3-acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) and a cytokinin Kinetin (K) either alone or in combination in varying concentrations. The pH of MS media was adjusted to 5.8 before autoclaving at 121°C for 20 min. The explants were inoculated in laminar air flow cabinet in the MS basal medium containing different concentration and combination of plant growth regulators. In this culturing stage, we evaluated plant growth regulators (PGRs) types and concentrations, basal medium composition for culture establishment, shoot proliferation and root regeneration (Table 2).

To observe the growth of explants fortified with various growth regulators the observations were recorded at two stages.

Stage (I) consisting of multiple shoot proliferation from axial buds after 30 days of initiating culture.

Stage (II) when the complete plants were obtained i.e. after root formation.

All observations were based on initial culture media, irrespective of regeneration medium.

a) Mode of regeneration/ 100 explants planted: the explants planted on different media were recorded for the percentage of explants showing direct shooting and percentage of plantlets. (stage I)

b) Plantlet regenerated/100 explant: Complete plantlet (shoot+root) regenerated from nodal explants directly. (stage II)

c) Callus induction: Induction of callus from nodal explants occurred was recorded for further experiments to be conducted in the future that is beyond the scope of current investigation.

2.3. Culture Conditions

The explants inoculated in the media were initially incubated in darkness in a culture chamber at 25°C ± 2°C. Subsequently, explants were incubated under a 16/8-h (light/ dark) photoperiod with light supplied by1250 lux of cool white fluorescent at a constant temperature of 25°C ± 2°C.

2.3. Rooting and Acclimatization

Regenerated shoots were excised and transferred to vessels containing 30 ml MS basal medium for root growth. MS basal medium with 3 different concentrations of indole- 3-acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) and Kinetin (K) either alone or in combination in varying concentrations with 3% sucrose and 0.1% agar were tested for initiation, regeneration and growth of roots from cultured shoots. For acclimatization *in vitro* plantlets, roots of the regenerated plantlets were washed well with sterile distilled water to remove agar and dipped in 0.5% bavistin for 5 min. Plantlets were transferred to pots containing soil, sand, vermicompost in a greenhouse for 40 days after which the plantlets were planted in college garden.

3. RESULT AND DISCUSSION:

Using Micropropogation technique, it has been possible to conserve *Rauwolfia serpentine* an endangered plant using tissue culture of nodal explants[8]. The application of 1% $HgCl_2$ solution was found to be most suitable method of disinfection and higher concentration of $HgCl_2$ caused high tissue damage resulting in death of explants within 48 to 72 hrs of culture inoculation on to the media. High frequency of plant regeneration protocol was standardized that was simple, efficient and time saving. Regeneration of axillary shoots was obtained on MS medium supplemented through different sets of hormonal combinations. This was done by using nodal explants of *Rauwolfia serpentine* in MS media supplemented with varied concentration of auxin and cytokinin (alone as well as in varying combination). It was observed that the combination of cytokinins and auxins (K and IAA) in MS media was ideal for axial shoot elongation. Four to six vigorously growing shoots were developed on the explant, which was due to the multiplication of the original bud. Multiple Shoot formation and elongation of axillary bud were obtained on MS supplemented with 2.5 mg/l K + 0.4 mg/l IAA. After shoot elongation these shoots were cut from mother explants and rooting was induced using a combination of auxin and cytokinin (IAA and K). Rooting ability of micro shoots obtained in the present studies was satisfactory. Highest rooting frequency was obtained in MS media containing 0.6 mg/l K + 2.0 mg/l IAA. The combination of hormones namely 2,4-D and K resulted in induction of callus. The combination of cytokinin and auxin that showed high percentage of callusing in MS media was 0.8 mg/l K + 1.8 mg/l 2,4-D (Table 3).

Table 1: Table 4.1- Standardization of sterilization treatment for *Rauwolfia serpentina* explants with various $HgCl_2$ concentratrions for three minutes. (TD) represent tissue death.

SN	Sterilization reagents	Explant 1	Explant 2	Explant 3	Explant 4
2	0.1% $HgCl_2$	-	-	-	-
1	0.3% $HgCl_2$	-	-	-	-
	0.5% $HgCl_2$	+	+	++	+
3	1% $HgCl_2$	+++	++++	++++	+++
4	1.5% $HgCl_2$	++	++	++	++
		TD	TD	TD	TD

Table 2: Experiments were conducted using two Auxins (IAA and 2,4-D) and Cytokinin (Kinetin) either alone or in combinations in varying concentrations.

S.N.	Culture media	Growth regulators mg/l		
		IAA	2,4-D	Kinetin
1	MS 00	-	-	-
2	MS 0.2K	-	-	0.2
3	MS 0.5 K	-	-	0.5
4	MS 1.0 K	-	-	1.0
5	MS 1.5K	-	-	1.5
6	MS 2.0 K	-	-	2.0
7	MS 2.5 K			2.5
8	MS 3.0 K			3.0
9	MS 0.2 IAA	0.2	-	-

10	MS 0.5IAA	0.5	-	-
11	MS 1.0 IAA	1.0	-	-
12	MS 1.5 IAA	1.5		-
13	MS 2.0IAA	2.0		-
14	MS 2.5 IAA	2.5		-
15	MS 3.0 IAA	3.0		-
16	MS 0.2 2,4-D	-	0.2	-
17	MS 0.5 2,4-D	-	0.5	-
18	MS 1.0 2,4-D	-	1.0	-
19	MS 1.5 2,4-D	-	1.5	-
20	MS 2.02,4-D	-	2.0	-
21	MS 2.52,4-D	-	2.5	-
22	MS 3.0 2,4-D	-	3.0	-
23	MS 1.5 K + 0.1 IAA	0.1	-	1.5
24	MS 1.8 K + 0.2 IAA	0.2	-	1.8
25	MS 2.0 K + 0.3 IAA	0.3	-	2.0
26	MS 2.5 K + 0.4 IAA	0.4	-	2.5
27	MS 3.0 K + 0.5 IAA	0.5	-	3.0
28	MS 3.5 K + 0.8 IAA	0.8	-	3.5
29	MS 1.5 K + 0.1 2,4-D	-	0.1	1.5
30	MS 1.8 K + 0.2 2,4-D	-	0.2	1.8
31	MS 2.0 K + 0.3 2,4-D	-	0.3	2.0
32	MS 2.5 K + 0.4 2,4-D	-	0.4	2.5

33	MS 3.0 K + 0.5 2,4-D	-	0.5	3.0
34	MS 3.5 K + 0.8 2,4-D	-	0.8	3.5
35	MS 0.1 K + 0.1 IAA	0.1	-	0.1
36	MS 0.2 K + 0.5 IAA	0.5	-	0.2
37	MS 0.3 K + 1.0 IAA	1.0	-	0.3
38	MS 0.4 K + 1.5 IAA	1.5	-	0.4
39	MS 0.6 K + 2.0 IAA	2.0	-	0.6
40	MS 0.8 K + 2.5 IAA	2.5	-	0.8
41	MS 0.1 K + 0.1 2,4-D	-	0.1	0.1
42	MS 0.2 K + 0.5 2,4-D	-	0.5	0.2
43	MS 0.3 K + 1.0 2,4-D	-	1.0	0.3
44	MS 0.5 K + 1.5 2,4-D	-	1.5	0.5
45	MS 0.8 K +1.8 2,4-D	-	1.8	0.8
46	MS 1.0K + 2.0 2,4-D	-	2.0	1.0

Table 3: Response of nodal explants cultured on MS medium supplemented with different growth regulators in varying concentrations.

S.N.	Culture media	Multiple Shoot formation and elongation of axillary bud.	Root Induction	Callus formation
1	MS 00	+	-	-
2	MS 0.2K	+	-	-
3	MS 0.5 K	+	-	-
4	MS 1.0 K	+	-	-

5	MS 1.5K	+	-	-
6	MS 2.0 K	+	-	-
7	MS 2.5 K	+	-	-
8	MS 3.0 K	+	-	-
9	MS 0.2 IAA	-	-	-
10	MS 0.5IAA	-	-	-
11	MS 1.0 IAA	-	-	-
12	MS 1.5 IAA	-	-	-
13	MS 2.0IAA	-	-	-
14	MS 2.5 IAA	-	-	-
15	MS 3.0 IAA	-	-	-
16	MS 0.2 2,4-D	-	-	-
17	MS 0.5 2,4-D	-	-	-
18	MS 1.0 2,4-D	-	-	-
19	MS 1.5 2,4-D	-	-	-
20	MS 2.02,4-D	-	-	-
21	MS 2.52,4-D	-	-	-
22	MS 3.0 2,4-D	-	-	-
23	MS 1.5 K + 0.1 IAA	+	-	-
24	MS 1.8 K + 0.2 IAA	+	-	-
25	MS 2.0 K + 0.3 IAA	++	-	-
26	MS 2.5 K + 0.4 IAA	+++	-	-
27	MS 3.0 K + 0.5 IAA	++	-	-

28	MS 3.5 K + 0.8 IAA	+	-	-
29	MS 1.5 K + 0.1 2,4-D	+	-	-
30	MS 1.8 K + 0.2 2,4-D	+	-	-
31	MS 2.0 K + 0.3 2,4-D	+	-	-
32	MS 2.5 K + 0.4 2,4-D	+	-	-
33	MS 3.0 K + 0.5 2,4-D	+	-	-
34	MS 3.5 K + 0.8 2,4-D	+	-	-
35	MS 0.1 K + 0.1 IAA	+	-	-
36	MS 0.2 K + 0.5 IAA	+	-	-
37	MS 0.3 K + 1.0 IAA	+	-	-
38	MS 0.4 K + 1.5 IAA	+	+	-
39	MS 0.6 K + 2.0 IAA	+	+++	-
40	MS 0.8 K + 2.5 IAA	+	++	-
41	MS 0.1 K + 0.1 2,4-D	+	-	-
42	MS 0.2 K + 0.5 2,4-D	+	-	-
43	MS 0.3 K + 1.0 2,4-D	+	-	+
44	MS 0.5 K + 1.5 2,4-D	+	-	++

45	MS 0.8 K +1.8 2,4-D	+	-	+++
46	MS 1.0K + 2.0 2,4-D	+	-	++

Response to hormones: Very High >80% (+++); High (++); Moderate 30-60 % (++); Low< 30%(+) and No effect (-).

CONCLUSION:

The present study suggests a rapid multiplication method for the propagation of *R. serpentina*, the critically endangered plant. It was observed that maximum multiple shoots induction from axiallary bud occurred in MS media containing 2.5 mg/l K + 0.4 mg/l IAA while root induction was best in MS media containing 0.6 mg/l K + 2.0 mg/l IAA and callus was induced in MS media containing 0.8 mg/l K +1.8 mg/l 2,4-D.Moreover, this simple and rapid propagation protocol is reproducible and can be useful in the future conservation programmes of *R. serpentina.* Thus the propagation of plants from axillary buds has proved to be the most generally applicable and reliable method of *in vitro* propagation in *Rauwolfia 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. It is hence concluded that the outlined procedure offers a potential and viable method to conserve and propagate *Rauwolfia serpentine* from nodal explants.

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