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A novel protocol for micropropagation of *Rauvolfia serpentina* : In low concentration of growth regulators with sucrose and phenolic acid

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SUMMARY

A novel *in vitro* propagation system for *Rauvolfia serpentina* (Apocynaceae family), a medicinally important plant has been developed. 200 alkaloids have been isolated from the plant. In this study *in vitro* plantlets were directly regenerated from the apical segments in MS medium supplemented with different concentration of IBA (0.125-0.5 mg/l) in combination with BAP (0.5-2.0 mg/l), which are far below the limits reported by other workers making this process cost effective. For multiplication of shoots MS media supplemented with BA (0.1 mg/l) and different concentration (0-50 g/l) of sucrose. Use of sucrose especially the higher concentration has never been attempted before and gave very positive results. The elongated and multiple shoot primordial sub-cultured on to rooting media using two-step pulse treatment method using IBA (25-100 μM) and phenolic acid (1%). Rooted plantlets were successfully transferred to the field, after acclimatization in the net house.

Key words : *Rauvolfia serpentina,* Micro propagation, Regeneration. Nodal segment, Growth regulator.

Rauvolfia serpentina (Indian snake root), an important medicinal shrub, belongs to the Apocynaceae family. *Rauvolfia* root is bitter, acrid, laxative anthelmic, diuretic and sedative .The alkaloids are classified in to 3 groups, *viz.*, reserpine, ajmaline and serpentine groups.

Reserpine group comprising reserpine, rescinnamine, deserpine group etc. Ajmaline group comprising ajmaline, ajmalicine, ajmalinine, isoajmaline etc. Serpentine group comprising serpentine, alsotonine etc. (Husain, 1993; Iyengar, 1985). The root is a sedative and is used to control high blood pressure and certain forms of insanity (Ghani, 1998). In Ayurveda it is also used for the treatment of insomnia, epilepsy, asthma, acute stomachache and painful delivery. It is used in snake bite, insect stings and mental disorders. Reserpine is a potent hypotensive and tranquillizer but its prolonged usage stimulates prolactine release and cause breast cancer. The juice of leaves is used as a remedy for the removal of opacities of cornea. (Baksha *et al*., 2007). The present communication report is use of low concentration of growth regulators, sucrose and phenolic acid for regeneration of *Rauvolfia serpentina*.

MATERIALS AND METHODS

The methods of plant tissue culture were the standard method as described in plant cell, tissue and organ culture fundamental methods (Gamborg and Phillips, 2004). The explants used for the *in vitro* propagation of *Rauvolfia* were nodal pieces of 0.7-0.8 cm collected from 3-4 month old mature plants growing in medicinal plants nursery of Bhopal. The explants were washed thoroughly first under running tap water for 30 min to remove adherent particles, then treated with a liquid detergent for 20 min followed by washing in tap water and rinsed 5 times with double distilled water with 1 % savlon and finally treated with $HgCl_2$ (0.1%) for 6 minute in a laminar flow cabinet and washed four times with autoclaved double distilled water to remove any trace of $HgCl_2$ solution.

Culture media and incubation:

The basal medium used in all experiments was Murashige and Skoog (1962) mineral formulation (MS) containing standard salts, vitamins, 3% (w/v) sucrose, $CaCl_2$(0.44 gm) and 0.8% (w/v) agar. MS medium was used for induction of shoots and MS half media was used for induction of roots. The pH of the medium was adjusted to 5.8 before gelling with agar and autoclaved at 121°C at 15 lbs pressure for 20 min. The surface sterilized explants were inoculated on the above medium under aseptic conditions.

Shoot initiation and multiplication media:

For shoot induction, apical segment explants were placed on MS medium with 6-benzyladenine (BA) at different concentrations (0.5, 1.0, 1.5, 2.0 mg/l) either singly or in combination with indole-3-butyric acid (IBA) at different concentrations (0.125, 0.25, 0.37, 0.5 mg/l).

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Multiple shoots induction were obtained on MS medium supplemented with 0, 10, 20, 30, 40 and 50 g/l sucrose in the presence of (0.1 mg/l) BA.

Two step rooting procedure with pulse treatment:

Induction of roots at the base of *in vitro* grown shoots is essential and indispensable step to establish tissue culture derived plantlets to the soil. The isolated micro shoots were rooted with 60-90% success in 4 weeks by a two-step culture procedure using a strategy of giving pulse treatment of an auxin IBA (25, 50, 75, and 100 μM) together with a phenolic acid (1%) for 1 week followed by transfer of such shoots to solid half strength MS medium without IBA and phenolic acid.

Acclimatization and transfer of plantlets to soil:

Cultures were maintained in a culture room under cool white fluorescent light (80-100μ mole photon m-2 s-1). Plantlets with well developed shoots and roots were removed from the culture medium, washed gently in running tap water and transferred to plastic pots. Pots containing garden soil mixed with vermiculite and sand (1: 1) under controlled growth chamber conditions (25± 2°C and 50-55% relative humidity). After 30 days, the plants were kept under shade for 2 weeks and then placed outdoor under natural light.

RESULTS AND DISCUSSION

For successful micro propagation shoot tip cultures are preferred. Shoot tip explants from field grown 3-4 months old mature plants of *Rauvolfia serpentina* were cultured on MS medium supplemented with various concentration of BA (0.5,1.0,1.5,2.0 mg/l)) and BA+IBA (0.125,.25, .37,.5 mg/l) (Table 1).

Cytokinins have been defined as substrates that stimulate cell divisions in plants and interact with auxin in determining the direction of cell differentiation (Warding and Phillips, 1981). In the present study when two cytokinines were used alone (without combination of auxin); it was observed that BA was more effective for shoot multiplication than Kn. Addition of exogenous auxin to the medium promoted apical shoot proliferation and enhanced the growth of culture. One of the advantages of adding auxin at low concentration on the culture media is to nullify the effect of the higher concentration of cytokinine on axillary shoot elongation (Hu and Wang, 1983). The process of root initiation, development and elongation normally requires the medium that contains auxins.

In the present study during one week after inoculation, bud initiation started in most of the cultures after 10 days (Fig. 1). For initiation of shoots cytokinin and auxin were tested. A combination of the BA (0.5 mg L^{-1}) + IBA (0.125 mg L^{-1}) proved to be the best which showed 5.9 shoot buds with 5 fold increase in shoot length (3.5) as compared to control ($p<0.01$). The second best result was found in MS medium supplemented with BA (1.0 mg L^{-1}) + IBA (0.25 mg L^{-1}) showing 5.3 shoot buds with 4.57 fold increase in shoot length (3.2) as compared to control ($p<0.01$).

Multiple shoot regeneration and elongation of shoot primordia started after two weeks of culture (Fig. 2). For this different concentrations of sucrose were used (Table 2). The best and rapid multiple shoot potentiality was observed on MS medium supplemented with BA (0.1 mg/l) + sucrose (40 g/l), which showed 12 multiple shoots with 7.55 fold increase in shoot length (6.8) as compared to control ($p<0.01$).

It was observed that proliferation and growth of cultures were strongly influenced by sucrose concentration. Significant interactions of sucrose concentration with cytokinine were observed for shoot

Table 1 : Effect of different concentration of BA and IBA in MS medium on shoot initiation through nodal explant of *Rauvolfia serpentina* (After one week)

Sr. No.	Growth regulators (mg L^{-1})	Number of shoots per explant	Shoot length (cm)	Survival rate %
1.	Control	-	0.7± 0.09	0 %
2.	MS + BA 0.5	1.9± 0.22	1.0± 0.18	55 %
3.	MS + BA 1.0	3.6± 0.39	2.2± 0.40	80 %
4.	MS + BA 1.5	4.9± 0.71	3.0± 0.51	90 %
5.	MS + BA 2.0	2.6± 0.31	1.5± 0.28	55 %
6.	MS + BA 0.5 + IBA .12	5.9± 0.63	3.5± 0.75	99 %
7.	MS + BA 1.0 + IBA .25	5.3± 0.59	3.2± 0.70	96 %
8.	MS + BA 1.5 + IBA .37	4.1± 0.83	2.5± 0.45	88 %
9.	MS + BA 2.0 + IBA .5	2.5± 0.15	1.9± 0.32	60 %

* Values are mean ± standard error of three replicates with ten cultures per replicate; data scored after three weeks.

* Control – Apical or Shoot tip segment explants remain green and fresh but failed to develop multiple shoots in growth regulator free MS medium.

Table 2 : Effect of growth regular (BA) and different concentrations of sucrose on multiplication of *R. serpentina* plant

Sr. No.	Growth regulators (mg L^{-1}) and sucrose (g L^{-1})	Number of shoots per explant	Shoot length (cm)	Survival rate %
1.	Control	-	.9± 0.1	0 %
2.	MS + BA 0.1 mg/l + Sucrose 0 g/l	2 ± 0.30	1.2± 0.40	55 %
3.	MS + BA 0.1 mg/l + Sucrose 10 g/l	5± 0.49	2.5± 0.70	80 %
4.	MS + BA 0.1 mg/l + Sucrose 20 g/l	8± 0.60	4.5± 0.85	90 %
5.	MS + BA 0.1 mg/l + Sucrose 30 g/l	3± 0.35	1.6± 0.50	55 %
6.	MS + BA 0.1 mg/l + Sucrose 40 g/l	12± 0.97	6.8± 1.10	99 %
7.	MS + BA 0.1 mg/l + Sucrose 50 g/l	10± 0.80	5.5± 0.92	98%

* Values are mean ± standard error of three replicates with ten cultures per replicate; data scored after three weeks.
* Control – Apical or shoot tip segment explants remain green and fresh but failed to develop multiple shoots in growth regulator free MS medium.

Table 3 : Effect of different concentrations of auxins (IBA) on root induction from *in vitro* raised micro shoots of *R. serpentina* in MS medium containing phenolic acid (1 %) followed by their transfer to hormone free half strength MS medium after 4 weeks of culture

Sr. No.	Growth regulator IBA (µm)	Number of roots per explant	Root length (cm)	Survival rate %
1.	25	-	0.5±0.03	0 %
2.	50	8.5±0.75	5.9±0.52	97 %
3.	75	4.5±0.58	2.5±0.66	78 %
4.	100	2.7±0.23	1.9±0.12	60 %

* Values are mean ± standard error of three replicates with ten cultures per replicate; data scored after three weeks

Fig.1 : Expansion of first two leaves (MS + BA + IBA)

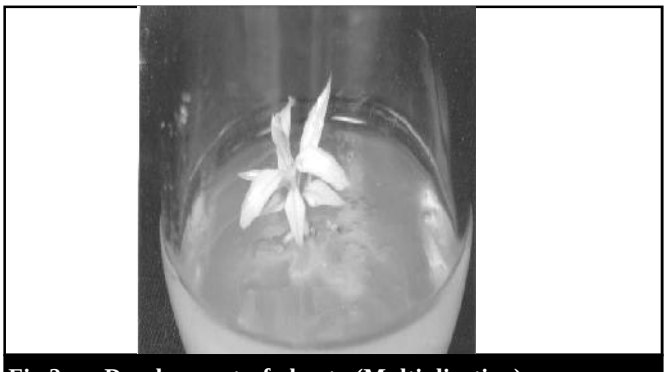

Fig.2 : Development of shoots (Multiplication)

height, bud number, and vigor. Increase in fresh (Deng and Donnelly, 1993; Romano *et al*., 1995) and dry weights (Capellades *et al*., 1991) of plantlets with increasing sucrose concentrations have been reported for other plants but no report is available for *Rauvolfia serpentine*. This increase in plantlets with increasing sucrose concentration may be responsible for corresponding decreases in photosynthesis. Stored assimilates might be providing an utilizable form of energy during acclimatization, improving survival of plantlets cultured on high levels of sucrose. Sucrose concentration alone had little or no effect on shoot initiation, shoot growth increased considerably with sucrose, reaching an optimum on media containing 20-40 g /l . Although carbohydrate requirements can vary depending on the phase of culture (Romano *et al*.,1995), a concentration of at least 20 g/l was required to optimize shoot proliferation , growth and rooting . alternative carbohydrates sources were not tested in these experiments , but improved growth of soybean (Saka *et al*.,1980) and *S.galegifolia* (Ermayanti *et al*., 1994) cultures were reported when fructose or glucose, respectively were substituted for sucrose.

Rooting of *Rauvolfia serpentina* in IBA and phenolic acid showed large number of rootlets branches, in the roots compare to normal plant which had very few rootlets (Fig. 3). The best rooting was observed with IBA (50µM) + Phenolic acid (1 %), which showed 8.5 multiple roots with 5.9 shoot length (Table 3).

The two step culture procedure for rooting has also been reported in *Pterocarpus marsupium* (Anis *et al*.,

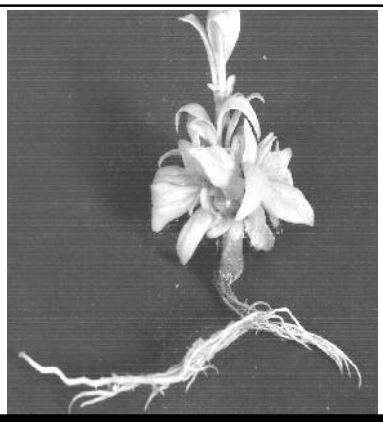

Fig. 3 : *Ex-vitro* root initiation (MS + IBA + Phenolic acid)

Fig. 4 : An acclimatized plant in pot

2005) and in *Hagenia abyssinica* (Feyissa *et al*., 2005) but the use of phenolic acid is a new concept for micro propagation of *Rauvolfia serpentina*. The incorporation of an auxin in the medium generally promotes rooting, while in the present study auxin alone in the MS medium was found to be ineffective for rooting.

Root formation is an energy demanding process and thus exogenous supply of carbohydrate is required. However, this being the last stage of *in vitro* culture, it is important to transfer the plant from heterotrophic nutrition to autotrophic nutrition (Fig. 4). Thus, the exogenous supply of sugar should be reduced at this time. The present protocol advocates the use of MS medium supplemented with BA and IBA for shoot induction, MS medium supplemented with BA and sucrose for multiplication of shoots and two step rooting procedures with pulse treatment for root induction of *Rauvolfia* shoot.

Reserpine is an important Indole alkaloid that is used to treat hypertension and various psychiatric diseases by acting as a tranquilizing agent. In pharmaceutical industries reserpine is in great demand, which is generally extracted from the roots. Chemical synthesis of reserpine is costlier than extracting it from natural resources. So extracting this alkaloid in the already available system is a beneficial approach. We recommend the use of low concentration of growth regulators, doudling the amount of sucrose and phenolic acid an unproved protocol for regeneration of *Rauvolfia serpentina*. This protocol is novel because of its minimal requirement and cost effectiveness for propagation.

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Abbreviations :

BA -6-benzylaminopurine
IBA- Indole butyric acid
MS- Murashige and skoog

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