Somatic embryogenesis and *in vitro* regeneration of an endangered medicinal plant sarpgandha (*Rauvolfia serpentina* L.)

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Abstract

Objective. An efficient protocol for *in vitro* regeneration of endangered medicinal plant *Rauvolfia serpentina* has been developed. *Methods and Results*. The juvenile leaf explants were transferred to MS medium containing different combinations of PGRs. Among the various combinations of BAP (1.0 – 3.0 mg/L) and IAA (0.1 – 0.5 mg/L) the intensity of callus induction was highest in 2.5 mg/L BAP + 2.0 mg/L IAA and 1.0 mg/L BAP + 0.5 mg/L IAA. The frequency of callus induction was highest 77.77% in 1.0 mg/L BAP + 0.5 mg/L IAA. During organogenic callus formation, different types of calli with variation in colour and texture were noticed and among them, the light green, fragile calli responded well for the induction of shoots. Among the various combinations of BAP and IAA used the frequency of shoot regeneration was highest 75% in 2.5 mg/L BAP + 0.4 mg/L IAA. For elongation of shoot 1 ppm GA-3 was also used, this provides a better result. The shoot was transferred to MS medium for root regeneration containing PGRs: BAP (2.5 mg/L) + IAA (0.3 – 0.5 mg/L) + NAA (0.3 – 0.5 mg/L). The frequency of root regeneration was 100% in MS medium containing BAP (2.5 mg/L) + IAA (0.5 mg/L) + NAA (0.5 mg/L). After rooting on shoots the plantlets were shifted to sterile soil field pots for acclimatization. The survival percentage of plants after hardening was 67%. *Conclusion*. The protocol was optimized by manipulations of different PGRs for enhanced multiplication. Protocol explained in this research paper provides a rapid plant regeneration system which could be used for the somaclonal variation; shoot induction and producing transgenic plants in *Rauvolfia* through *Agrobacterium* and biolistic methods. [Life Science Journal. 2009; 6(2): 57 – 62] (ISSN: 1097 – 8135).

Keywords: *Rauvolfia serpentina*; regeneration; *in vitro*; induction

1 Introduction

Medicinal plants have been the subjects of man's curiosity since time immemorial (Constable, 1990). Almost every civilization has a history of medicinal plant use (Ensminger *et al*, 1983). Approximately 80% of the people in the world's developing countries rely on traditional medicine for their primary health care needs, and about 85% of traditional medicine involves the use of plant extracts (Vieira and Skorupa, 1993). *In vitro* cell and tissue culture methodology is envisaged as a mean for germplasm conservation to ensure the survival of endangered plant species, rapid mass propagation for large-scale revegetation, and for genetic manipulation studies. Combinations of *in vitro* propagation techniques (Fay, 1992) and cryopreservation may help in conservation of biodiversity of locally used medicinal plants.

Rauvolfia serpentina L. commonly known as sarphgandha is an important medicinal shrub of family Apocynaceae (Nathan Kline, 1954). The snake-weed genus includes about 50 species, this has fairly wide area of distribution, including the tropical part of the Himalayas, the Indian peninsula, Sri Lanka, Burma, and Indonesia. The plant is indigenous to India, Bangladesh and other regions of Asia and found to grow in the wild in many places around the country (Ghani, 1998). Its roots contain 0.15% resperine-rescinnamine group of alkaloids (Anonymous, 1969). It also contains a number of bioactive chemicals, including ajmaline, deserpidine, rescinnamine and yohimbine (Lewis WH, 2003). This herbal plant is used as medicine for high blood pressure, insom-

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nia, anxiety and other disorders of the central epilepsy (Ghani, 1998).

Rauvolfia is threatened in India due to indiscriminate collection and over exploitation of natural resources for commercial purposes to meet the requirements of pharmaceutical industry, coupled with limited cultivation (Nayar and Sastry, 1987; Gupta, 1989). IUCN has kept this plant under endangered status. The chemical resperine is an alkaloid first isolated from roots of *Rauvolfia serpentina* and is used to treat hypertension (Ford RV *et al*, 1953; Vida F, 1953). Although, for centuries they have been used empirically in India for a variety of conditions that they were effective in relief of hypertension was first commented on by Bhatia in 1942. Subsequently, other clinical investigators working in India confirmed the effectiveness of *Rauvolfia serpentina* for that purposes (Chakraverti NK *et al*, 1951; Gupta JC, 1942). In a short term study, a significant decrease in systolic as wall as diastolic blood pressure of patients to whom the drug was given was observed (Vakil, 1949). Insanity, Snakebite and Cholera can also be treated by use of this alkaloid (Wild R, 1994). The pectic polysaccharide named rauvolfian RS was obtained from the dried callus of *Rauvolfia serpentina* L. by extraction with 0.7% aqueous ammonium oxalate and it was found to posses some anti-inflammatory effect (Popov SV *et al*, 2007).

In approximately 60% of medicinal plants used in traditional medicines, roots are the principal source of drug preparation (Kamboj, 1988). The development of fast growing culture system can offer an opportunity for producing drugs from the roots in the laboratory with out having to depend solely on field cultivation (Sudha and Seeni, 2003).

In vitro regeneration of sarphgandha has been done from several genotypes. Micropropagation has been achieved from explant of *Rauvolfia micrantha* Hook F cultures (Sudha and Seeni, 1996). Micropropagation can be considered as an important tool for the production of higher quality plant based medicines (Debnath M, 2006). In view of this, there is an urgent need to apply *in vitro* culture methods for the micropropagation and conservation of this valuable endangered plant. Here efforts have been made to define efficient protocol for the recovery of plants through organogenesis of Rauvolfia serpentina. *In vitro* regeneration of *Rauvolfia* has been reported by many authors (Butenka, 1964; Mitra and Kaul, 1964; Vollosovich and Butenka, 1970; Kukreja *et al*, 1989; Roy *et al*, 1994). The present study was undertaken to develop a more efficient protocol for rapid in vitro multiplication of *Rauvolfia serpentina* using leaf explant as an initial plant material.

2 Materials and Methods

2.1 Plant material

Plantlets of *Rauvolfia serpentina* were obtained from Corbett Jadibuti Udhyan Kaladhungi, Nainital, Uttarakhand and grown in sterile vermiculite at 25 ºC – 30 ºC in light. All the explants were taken from these donor plants for present investigation. Leaf explants from 2 months old donor plant was kept for 2 hours in systemic fungicide Bavistin (VIMCO pesticides, Gujarat) and Tween-80, an antimicrobial agent, prior to surface sterilization. For surface sterilization, chemicals such as $HgCl_2$ (0.1%), NaOCl (1%), H_2O_2 (1%) and ethanol (70%) was used. Juvenile leaves were washed thoroughly in running tap water for 30 minutes and then with distilled water three times. Leaves were treated with bavistin solution for 4 – 5 minutes, and then rinsed thoroughly with sterile distilled water. The leaves were subjected to 0.1% $HgCl_2$ for 30 seconds, washed with distilled water and then placed in 70% ethanol for 1 minute and again washed with distilled water, followed by addition of three drops of antibiotic solution (cefotaxime) in laminar airflow cabinet. In the antibiotic solution, all leaves were dissected into small pieces and treamed so that maximum part can be exposed to media. All the chemicals used were purchased from Hi-media unless stated otherwise.

2.2 Culture media and growth condition

The medium comprised of macro and micro elements according to Murashige and Skoog (1962) with mesoinosotol (100 mg/L), thiamine-HCl (0.5 mg/L), pyridoxine-HCl (1 mg/L), nicotinic acid (0.5 mg/L) and sucrose (30 g/L), solidified with 0.6% agar. The plant growth regulators used were 6-benzyl-aminopurine (BAP), α-naphthalene acetic acid (NAA) and indole acetic acid (IAA). All experiments were carried out in culture tubes (150 × 25 mm) containing 30 ml of culture medium. The pH of media were adjusted to 5.8 prior to autoclaving at 121 ºC at 15 lbs pressure for 20 minutes. Cultures were incubated under 16 hours/8 hours light/dark cycles (artificial light, 80 μM per m^2/s).

2.3 Callus induction and shoot regeneration

For callus induction juvenile leaf section (3 – 5 mm in length) with cut end surface in contact with culture medium were placed on MS medium supplemented with various concentrations of PGRs BAP and IAA. After 20 days of culture, the leaves cultured on MS basal medium supplemented with 3% (w/v) sucrose, BAP (1.0 ppm) and IAA (0.5 ppm) were found to give profuse callusing and when callusing was observed in entire explant, the

callus was cut into small pieces transferred to MS media having BAP and IAA in same concentration as for callus induction. Subculturing was done after every 1 – 2 weeks. After 3 – 4 weeks of subculturing first shooting is observed in callus.

2.4 Regeneration of roots and development of complete plantlets

For initiation of roots the 6 – 8 weeks old shoots (2.5 – 4.0 cm in length) were cultured on half strength MS basal medium supplemented with 2% (w/v) sucrose and different concentration of PGR were tested BAP (2.5 ppm) : IAA (0.3 ppm) : NAA (0.3 ppm), BAP (2.5 ppm) : IAA (0.4 ppm) : NAA (0.4 ppm), and BAP (2.5 ppm) : IAA (0.5 ppm) : NAA (0.5 ppm), for 2 – 3 weeks. The shoots were also tested on hormone free full and half strength MS basal medium with 3% sucrose (w/v) for root initiation.

The complete rooted plantlets (6 – 10 weeks old) were washed free of agar and dipped in 0.2% bavistin fungicide for 5 – 10 minutes, and potted in small plastic pots containing sterilized soilrite. The plantlets were covered with polythene bags to maintain high humidity. These were acclimatized at 25 ± 3 ºC less than 16 hours photoperiod and watered regularly. After 3 – 4 weeks, the polythene bags were removed and established plantlets were transplanted to earthen pots in a greenhouse.

3 Results and Discussion

The smaller size of explants were chosen due to fact that smaller size of explants provide less chance of contamination, as well as longer leaves showed total loss of morphogenic potential (Mujib A, 2003). Initiation of calluses from leaf explants did not pose a major problem. During initiation the explants did not show any leaching or browning of tissues. MS basal medium was the most effective for callusing of leaf explants. The explants cultured on MS basal medium supplemented with different combinations of BAP and IAA showed varied response for callusing (Table 1). Leaf explants culture on MS basal medium without any PGR supplementation showed only swelling of explants that were not significant for callusing. This was possibly due to significant role of PGR over callusing. In the media supplemented with BAP and IAA, the leaf segments remain green for long period with very slow process of callus induction (Figure 1). Further transfer into media containing BAP and IAA rapidly showed callus induction because the excretion of phenolic compounds from explants to the medium was strictly avoided by regular sub-culturing of callus (Figure 2).

Callus is an unorganized mass of plant cells and its formation is controlled by growth regulating substances present in the medium (auxins and cytokinins) (Shah *et*

Table 1. Effect of different concentrations of PGR added to MS medium on induction of callus from leaf in *R. serpentina* (Observation after 27 days)

PGR (mg/L)		Intensity of callus induction	Nature of callus
IAA	BAP		
0.1	–	–	No callus formation
0.1	1.0	++	White coloured, fragile
0.1	1.5	+	Green coloured, fragile
0.2	0.0		No callus formation
0.2	1.0	++	Light green coloured, fragile
0.2	1.5	+	Light green coloured, fragile
–	2.0	–	No callus formation
–	2.5	–	No callus formation
–	3.0	–	No callus formation
0.1	2.0	+	White coloured, fragile
0.1	2.5	–	No callus formation
0.1	3.0	–	No callus formation
0.2	2.0	–	No callus formation
0.2	2.5	+++	White coloured, fragile
0.2	3.0	–	No callus formation
0.3	–	–	No callus formation
0.3	1.0	++	Green coloured, fragile
0.3	1.5	+	Light green coloured, fragile
0.3	2.0	–	No callus formation
0.3	2.5	++	Light green coloured, fragile
0.3	3.0	–	No callus formation
0.4	–	–	No callus formation
0.4	1.0	+	Light green coloured, fragile
0.4	1.5	+	Light green coloured, fragile
0.4	2.0	–	No callus formation
0.4	2.5	++	Light green coloured, fragile
0.4	3.0	–	No callus formation
0.5	–	–	No callus formation
0.5	1.0	+++	Light green coloured, fragile
0.5	1.5	+	Green coloured, fragile
0.5	2.0	++	Light green coloured, fragile
0.5	2.5	–	No callus formation
0.5	3.0	–	No callus formation
–	–	–	Swelling of the explant observed

al, 2003). The specific concentration of plant regulators needed to induce callus, varies from species to species and even depends on the source of explant (Charriere *et al*, 1999). It has been demonstrated in many cases that 2,4-D is usually the choice of auxin for callus induction and subculture of grasses (Bhaskaran and Smith, 1990; Chaudhury and Qu, 2000). Lately more and more experimental results indicate that the addition of a low concentration of cytokinin in callus culture medium often enhances callus regeneration (Alpeter and Posselty, 2000; Chaudhury and Qu, 2000; Cho *et al*, 2000; Bai and Qu, 2001; Bradely *et al*, 2001). Minimal cytokinins and auxins in culture media would avoid somaclonal variation and efficiently produce true to type plantlets (Edson *et al*, 1996).

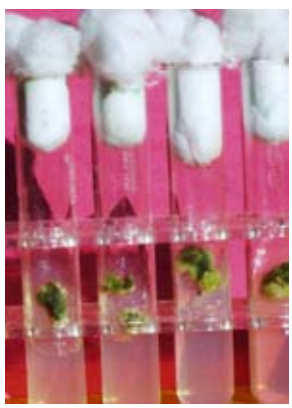

Figure 1. Callus induction in *R. sepentina* from leaf explants in MS media containing 1.0 mg/L BAP and 0.5 mg/L IAA.

Figure 2. Shoot regeneration from callus on 2.5 mg/L BAP + 0.4 mg/L IAA MS media.

The success of micropropagation largely relies on the selection of suitable plant part, which is to be used as the starting material for the experiment. In the present experiment leaf explants was best fit for purposes. The best callusing was observed in media having BAP : IAA in concentration ratio of (1.0 : 0.5 ppm). In the media supplemented with only BAP and IAA the callus induction was very significant (Figure 2). This remains in accordance with previous reported work of (Mathur *et al*, 1987). Different types of calli with variation in colour and texture were noticed (Table 1) and among them, the light green, fragile calli responded well for the induction of shoots.

This study further demonstrates that shoot regeneration from callus was very earlier in media supplemented with BAP and IAA in concentration ratio of (2.5 : 0.4 ppm), in comparison to 2.5 : 0.3, 1.0 : 0.5 or 2.0 : 0.5 (Table 2 and Figure 3). Thus, the PGR concentrations have significant impact on shoot regeneration. This is basically due to endogenous level of growth regulators. For elongation of shoot 1ppm GA-3 was also used, this provides a better result (Figure 4).

Table 2. Effect of different concentration of PGRs added to MS medium on induction of callus and regeneration of shoots from leaf of *R. serpentina*

PGR (mg/L)		Days for callus formation (day)	Days of shoot regeneration after callusing (day)	Frequency of callusing (%)	Frequency of shoot regeneration (%)
BAP	IAA				
2.5	0.4	24	18	72.00	75
2.5	0.3	24	36	40.00	45.03
1.0	0.5	24	37	77.77	52
2.0	0.5	24	38	70.00	39.45

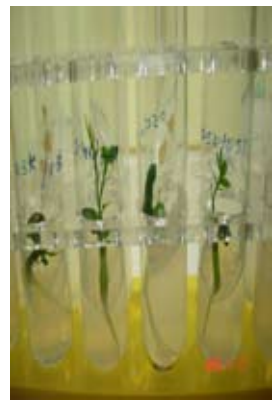

Figure 3. Rooting regeneration on 2.5 mg/L BAP + 0.5 mg/L IAA + 0.5 mg/L NAA MS media.

No root could be induced in either basal medium of full or half strength MS media. However, when 2.5 – 4.0 cm elongated shoots were placed on half strength MS

basal medium supplemented with BAP, IAA and NAA in concentration ratio of (2.5 : 0.5 : 0.5 ppm) roots were induced in nearly 100% of shoots within 2 weeks (Figure 5). Other concentration BAP, IAA and NAA (2.5 : 0.4 : 0.4 and 2.5 : 0.3 : 0.3) induce rooting in slightly lower percentage (Table 3). Basal media supplemented with NAA was found to be better for root regeneration this was in accordance with previous reported work of Kumar *et al* (1993).

Figure 4. *In vitro* regeneration of complete plantlets of *R. serpentina* from leaf explant.

Figure 5. Hardening of plantlet to mixture of sterile soil, sand and vermicompost.

Taking care of root regeneration data it can be concluded that the standard protocol developed for regeneration of *Rauvolfia* was nearly 100% efficient but in accordance with hardening data (Table 4) there is a need for further standardization and work to increase the efficiency, during hardening so that this medicinally important plant could be propagate at larger scale and its medicinal importance properties could be utilized for well being of human population. This further become important due to advancement in commercialization of plant tissue cultured plantlets by commercial sectors have led to continued exponential growth within the industry in terms of numbers of new units as well as numbers of plants produced by the units (Govil S and Gupta SC, 1997). The development of a reliable *in vitro* protocol are of great importance for producing plant material and for conservation of rare plant species, and offset the pressure on the natural populations as well as plant medicinal purposes.

Table 3. Effect of different concentration of PGR added to MS medium for root regeneration from shooted callus of *R. serpentina*

PGR (mg/L)			Days of rooting (day)	Frequency of rooting (%)
BAP	IAA	NAA		
2.5	0.3	0.3	12	85
2.5	0.4	0.4	15	96
2.5	0.5	0.5	10	100

Table 4. Estimated survival of plants after hardening

Number of pots containing plants	Number of plants survived	Percentage of survival (%)
5	3	60
3	2	67
4	2	50

4 Conclusion

The present study describes a well documented and reliable protocol of *R. serpentina* from leaf explants with much higher rate of multiplication. This protocol can be used as a basic tool for commercial cultivation of sarphgandha plant.

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