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Original Research Paper

In vitro regeneration and conservation of an endangered medicinal plant sarpagandha (*Rauvolfia serpentina* L.)

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ABSTRACT

An investigation on *in vitro* plant regeneration of endangered medicinal plant *Rauvolfia serpentina* L. was carried out. The newly emerging leaves were used as explants, which were transferred to half strength MS medium along with various combinations of growth regulators for callus regeneration. The half strength MS medium fortified with 5.0 mgl^{-1} 2,4-D and 2.0 mgl^{-1} NAA was found most suitable for qualitative (light green colour) and quantitative callus production (86%). The shoot regeneration (81.67 %) and elongation (5.67 cm) was highest in full MS medium supplemented with 6.0 mgl^{-1} BAP and 2.0 mgl^{-1} GA_3 and consequently, the root initiation was highest (55%) in half strength MS medium containing 3.0 mgl^{-1} IBA. The regenerated plantlets were preserved for nine months in good condition through repeated subculturing on full strength MS media. The callus were kept in storage by repeated sub-culturing in *in vitro* condition on MS medium fortified with 0.2 mgl^{-1} 2,4-D and the preservation could be extended for nine months in its best condition. It was evident that, the regeneration capacity of callus was reduced as the time of callus storage was increased.

Key words: *In vitro*, regeneration, *Rauvolfia*, callus, medicinal plant

INTRODUCTION

Rauvolfia serpentina L. commonly known as sarpagandha is a woody perennial medicinal plant belonging to family *Apocynaceae*. The roots of this shrub have been used in ayurvedic medicines from ancient time of Indian medical therapy (Singh *et al*., 2015). This shrub is highly effective for high blood pressure control. According to Ayurveda, it is the best among all anti-hypertensive drugs. It has been stated that, the drug is useful in mental disease, epilepsy, sleeplessness and several other ailments (Ojha and Mishra, 1985).

But due to increasing anthropogenic activities and rapidly eroding natural ecosystem, the natural habitat of this species is decreasing. *Rauvolfia* is threatened in India due to its indiscriminate collection and over exploitation of natural resources for commercial purposes to meet the requirements of pharmaceutical industry, coupled with limited cultivation (Singh *et al*., 2009). IUCN has kept this plant under endangered status (Anonymous, 2009; Jain *et al.*, 2003). In the present study, an attempt has been made to preserve the plant and to maintain its essence for future generation, as we know the plant is an endangered species.

Seeds of *Rauvolfia* has erratic and low germination percentage and if the seeds stored more than 7-8 months practically don't germinate at all. More over, the germination percentage of seed is very poor and variable ranging from 25-50 percent (Mitra, 1976). Hence, tissue culture technique in this plant is expected to be helpful to *ex situ* conservation by reducing the risk due to natural vagaries. Micropropagation is a unique method for

the production of homozygous and disease free plants. This technique would also facilitate to obtain large number of plants irrespective of season and an alternative source of secondary metabolites in colossal quantity. The process of micropropagation using various phytohormones in different concentration for *Rauvolfia* has been reported earlier (Roy *et al.*, 1994; Vanisree *et al.*, 2004; Baksha *et al*., 2007; Rahman *et al.*, 2008, Mallick *et al*., 2012).

But, the conservation of callus for months through indirect regeneration and mass replication of secondary metabolite in hassled free cultivation method is a new invention and there is also a need to apply *in vitro* methods for the regeneration and conservation of this valuable endangered plant. Hence, an effort has been made to develop an efficient protocol for the recovery of plants through organogenesis of *Rauvolfia serpentina*. It was hypothesised that, use of suitable plant growth regulators (PGR) as supplement of basic MS media could improve the *in vitro* plantlet regeneration and conservation potentiality of *Rauvolfia*. Different growth regulators as supplement of MS medium were optimized for rapid *in vitro* multiplication and conservation of *Rauvolfia serpentina* callus using leaf explant as an initial plant material.

MATERIAL AND METHODS

Plant material and explant preparation

The present investigation was carried out at the Tissue Culture Laboratory of the Department of Floriculture, Medicinal and Aromatic Plants, UBKV, West Bengal, India. The disease free juvenile leaf explants, used for callus formation and regeneration of *Rauvolfia serpentina* L. were collected from the green house of respective department of the university. They were washed three times with de-ionized water. Rest of the sterilization process was carried out in the horizontal laminar flow cabinet. The leaf was carefully made into 1 cm^2 incision along with the midrib. The excised explants were treated with different concentration of various disinfectants (4% Bavistin for 10 min and 0.1% $HgCl_2$ for 1 min.) and then cultured on the suitable medium. During inoculation, the basal portions of the leaves were kept in the contact with media.

Culture medium and experimental treatments

The medium comprised of macro and micro elements according to Murashige and Skoog (1962) with and sucrose of 30gl^{-1} (Himedia® Mumbai), solidified with 0.6% agar (Himedia® Mumbai). The Plant growth regulators used were 2,4-D, BA, NAA and IBA (Sigma Eldritch® USA). All experiments were carried out in 100 ml culture tubes and jam bottles (Borosil, Kolkata). The pH of media was adjusted to 5.7 prior to autoclaving at 121^{0}C at 15 lbs/sq inch pressure for 20 minute. Culture was incubated under 1600 lux light intensity for 16 h/8h light and dark cycles.

The leaf was placed on standard and ½ MS medium supplemented with various concentrations of 2,4-D (2.0 and 5.0 mgl^{-1}) and NAA (2.0 mgl^{-1}) for callus induction, growth and development (Table 1) . After 28 days of culture, the inducted *in vitro* calli were cut into small pieces and transferred to the medium for shoot regeneration. MS medium containing different concentration and combination of BAP (2.0, 4.0 and 6.0 mgl^{-1}) with GA_3 (2.0 mgl^{-1}) were used for shoot regeneration and shoot multiplication (Table 2). The shoot regeneration was observed after 3-4 weeks of subculturing. For initiation of roots 4-5 weeks old shoots (2-4 cm length) were cultured on half strength MS medium supplemented with different concentrations of IBA (1.0, 2.0 and 3.0 mgl^{-1}) (Table 3).

Callus conservation

The developed callus and regenerated plantlets calli were kept in *in vitro* condition under a foresaid temperature, light and humidity ideal for the plant development for a period of 9 months. The inducted calli were subcultured at 1½ month interval repeatedly on MS medium supplemented with 2,4-D (0.1, 0.2 or 0.3 mgl^{-1}) for regeneration of callus in *in vitro* (Table 4). Wherein, the *in vitro* regenerated plantlets were kept by subculturing at 1½ month intervals on different strengths of MS medium (full, ½ and ¼ MS) without any plant growth regulators (Table 5). The well developed callus were kept at 20±3°C under 1600 lux light intensity of 16h/8h day-night photoperiodic conditions and maintained as such for nine months by repeated sub culturing.

Table 1. Effect of 2, 4-D and NAA on callus induction in *Rauvolfia serpentina*

Sl. No.	Medium + Growth regulators (mgl^{-1})	% callus initiation	Days to callus formation	Callus morphology
1.	MS + 2.0 2,4-D	54.33 (47.47)*	23.67	White coloured, fragile
2.	MS + 5.0 2,4-D	62.00 (51.94)	21.33	Light green coloured , fragile
3.	MS + 5.0 2,4-D + 2.0 NAA	69.33 (56.35)	20.00	Green coloured , compact
4.	½ MS + 2.0 2,4-D	62.66 (52.30)	21.00	White coloured, fragile
5.	½ MS + 5.0 2,4-D	76.00 (60.67)	20.33	Light brown coloured , compact
6.	½ MS + 5.0 2,4-D + 2.0 NAA	86.00 (68.03)	18.33	Light green coloured , compact
SEm±		1.63	0.76	
CD (*P*=0.05)		5.09	2.36	

*"Arc sin transformed

MS - Murashige & Skoog

Table 2. Effect of BAP and GA_3 on shoot regeneration in *Rauvolfia serpentina*

Sl. No.	Medium + Growth regulators (mgl^{-1})	% shoot regeneration	Days to shoot regeneration	No. of shoots /plant	Shoot length (cm)
1.	MS + BAP 2.0 mgl^{-1}	45.00 (42.13)	32.667	1.333	2.00
2.	MS + BAP 4.0 mgl^{-1}	56.67 (48.79)	27.667	1.667	2.33
3.	MS + BAP 6.0 mgl^{-1}	60.00 (50.77)	25.667	2.000	3.00
4.	MS + BAP 2.0 mgl^{-1} + GA_3 2.0 mgl^{-1}	64.33 (53.31)	28.000	2.000	4.33
5.	MS + BAP 4.0 mgl^{-1} + GA_3 2.0 mgl^{-1}	76.33 (60.87)	25.667	3.333	4.67
6.	MS + BAP 6.0 mgl^{-1} + GA_3 2.0 mgl^{-1}	81.67 (64.60)	22.000	4.000	5.67
SEm±		1.831	1.800	0.333	0.27
CD (*P*=0.05)		5.704	5.608	1.038	0.85

MS - Murashige & Skoog

Table 3. Effect of IBA on root initiation in *Rauvolfia serpentina*

Sl. No.	Medium + Growth regulators (mgl^{-1})	% root initiation	Days to root initiation	No. of roots /plant	Root length (cm)
1.	½ MS + 1.0 IBA	40.00 (39.23)	16.333	2.000	2.00
2.	½ MS + 2.0 IBA	46.67 (42.94)	14.000	2.433	3.00
3.	½ MS + 3.0 IBA	55.00 (47·87)	12·000	3·000	4·67
SEm±		2.546	0.509	0.019	0.19
CD (*P*=0.05)		8.981	1.796	0.068	0.68

MS - Murashige & Skoog

Table 4. Effect of 2, 4-D on *in vitro* callus regeneration in *Rauvolfia serpentina*

Sl. No.	Medium + Growth regulators (mgL^{-1})	No. of Subcultures	Callus growth characteristics
1.	MS + 0.1 2,4-D	6	White and green callus became light brown and grow in size slowly
2.	MS + 0.2 2,4-D	6	Light green callus turn brown, increase in size and shape
3.	MS + 0.3 2,4-D	6	Light green fragile callus turn compact, later became brown

MS - Murashige & Skoog

Table 5. Effect of media strength on *in vitro* regeneration of plantlet in *Rauvolfia serpentina*

Sl. No.	Medium without growth regulators	No. of Subcultures	Plantlet growth characteristics
1.	MS	6	Growth and development with increased number of shoots and shoot length
2.	½ MS	6	Growth occurred and some root develop with plant drying
3.	¼ MS	6	Growth occurred and leaves became yellow with plant drying

MS - Murashige & Skoog

Acclimatization

The well rooted *in vitro* regenerated plantlets were transferred to plastic cups containing autoclaved vermiculite and vermicompost (1:1) for four weeks in culture room. Then the primary hardened plantlets were transferred to poly cups containing garden soil, sand, vermicompost (1:1:1) along with biofertilizers and were maintained under shed net with natural day length and temperature condition.

Experimental design and data analysis

The experiments were designed in completely randomized design (CRD). In each treatment 10 explants (n=10) were inoculated inside jam bottles separately. The statistical analysis was done by O.P. Stat software packages and the mean were compared using Duncan's multiple range test at the 5 % probability level.

RESULTS AND DISCUSSION

Callus regeneration

The callus grew within a month from inoculation on the callus formation medium containing different combinations of plant growth regulators viz. 2,4-D and NAA (Table 1). Among the different combinations ½ MS media supplemented with 5.0 mgl^{-1} 2,4-D and 2.0 mgl^{-1} NAA was found to be the best for maximum callus initiation (86.0%) of green coloured compact callus and took minimum days (18.33 days) to callus formation compared to others. It was also evidenced by Mallick *et al*. (2012) that, the high-frequency callusing induced in leaf and stem explant of *Rauvolfia serpentina* on modified MS medium supplemented with 2.5 mgl^{-1} 2,4-D. MS medium containing 2.0 mgl^{-1} of BAP plus 1.0 or 2.0 mgl^{-1} 2,4-D resulted into copious callus induction observed by Ilahi *et al*. (2007).

Shoot regeneration from callus

It was obtained from MS medium supplemented with different combinations of BAP and GA_3 (Table 2). MS medium supplemented with 6.0 mgl^{-1} BAP and 2.0 mgl^{-1} GA_3 gave highest level of shoot regeneration (81.67%) and number of shoots (4.0) per explant with a mean shoot length of 5.67 cm followed by the MS medium supplemented with 4.0 mgl^{-1} BAP and 2.0 mgl^{-1} GA_3 resulted better shoot length (4.67 cm) and higher number of shoots per explant (3.33). The data also reveals that, a combination of BAP and GA_3 is

necessary for shoot regeneration and elongation and also for early shoot development (22.00 days). Mallick *et al*. (2012) also reported that, the maximum regeneration of shoots from callus (90%) was observed in MS medium supplemented with 0.2 mgl^{-1} NAA and 1.5 mgl^{-1} BA. Similar result was obtained by Panwar *et at.* (2011) with maximum shoots (25.4) per culture from the callus in shooting medium containing 2.0 mgl^{-1} BAP + 0.5 mgl^{-1} NAA. Susila *et al.* (2013) showed that the best shoot proliferation (92%) was in MS medium containing 0.1 mgl^{-1} NAA and 2.5 mgl^{-1} BA.

Root regeneration

Out of different IBA concentrations tested, IBA 3.0 mgl^{-1} was the best for root initiation (Table 3). The root initiation was 55% at the same concentration. The total number of roots per explant was 3.0 with root length of 4.67 cm within 12 days of transferring. MS medium supplemented with IBA (2.0 mgl^{-1}) also induced better rooting (46.0%) with 2.43 roots per explant. The present results are in accordance with the result reported by Rahman *et al.* (2008) that, the adventitious shoots best rooted on half strength MS medium supplemented with 1.0 mgl^{-1} each of IBA and IAA. Rooting of the young shoots was obtained with 1.0, 2.0 or 4.0 mgl^{-1} of IBA (Ilahi *et al*., 2007). Susila *et al.* (2013) reported that half strength MS medium supplemented with 0.4 mgl^{-1} NAA and 0.1 mgl^{-1} IBA caused for maximum root formation (91%).

Callus conservation

The calli were best conserved by repeated sub-culturing on MS medium fortified with 0.2 mgl^{-1} 2,4-D evidenced with light green callus turn brown, increase in size and shape and the preservation could be extended for nine months in its best condition (Table 4 & Fig. 1D). The regeneration capacity of the stored callus was tested at three months interval by transferring the callus on best regeneration media. The maximum regeneration (72.0%) of the stored callus was noticed after three months of preservation with minimum days (13.67) taken for regeneration (Table 6). After nine months, the regeneration capacity of the repeated sub-cultured callus was reduced drastically with average of only 43.0% regeneration and the time taken for regeneration was longer (24.33 days). Significant results were found during callus conservation in terms of the capacity of callus regeneration. Furthermore, the plantlets regenerated from calli were survived successfully for nine months in good condition on full strength MS media comparing to ½ or ¼ strength media through repeated subculturing at 1½ month interval (Table 6 & Fig. 1E). This technique provides a large number of disease free true to type plantlets throughout the year which will confer a large scale cultivation of this endangered species for commercial cultivation. Therefore, this micropropagation protocol and *in vitro* conservation of *R. serpentina* will be useful for further improvement of this important medicinal shrub.

Table 6. Regeneration capacity of the callus in *Rauvolfia serpentina*

Sl. No.	Age of callus (months)	Regeneration (%)	Days to regeneration
1.	3	72.00 (58.05)	13.67
2.	6	54.67 (47.64)	18.00
3.	9	43.00 (40.98)	24.33
	SEm±	3.732	1.09
	CD (*P*=0.05)	13.165	3.84

(Figures in the parenthesis are angular transformation values)

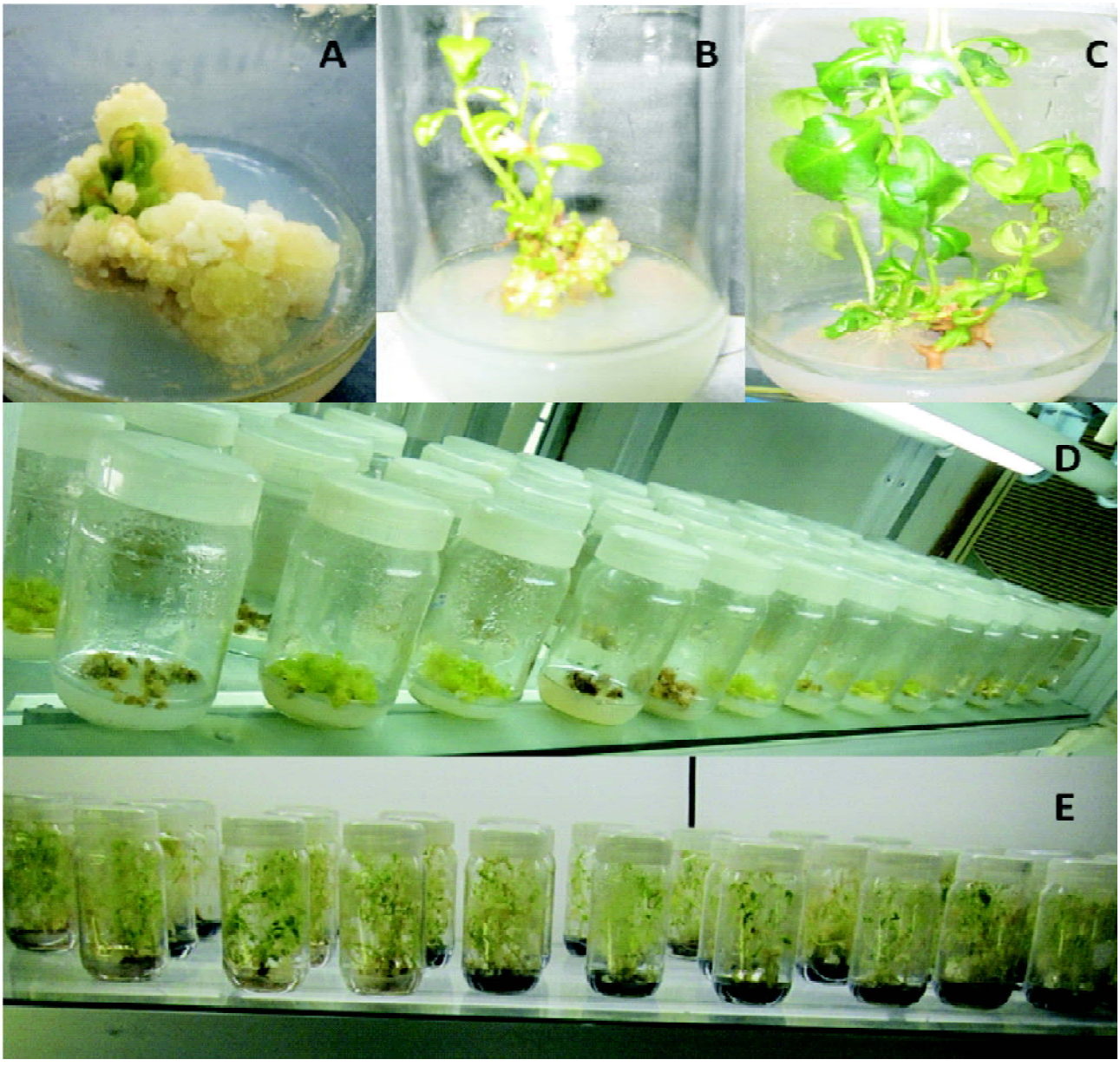

Fig. 1: *In vitro* regeneration of *R. serpentina*. A. Callus regenerated in ½ MS medium supplemented with 2,4-D 5.0 mgl^{-1} and NAA 2.0 mgl^{-1}. B. Multiple shoots induction n MS media containing BAP 6.0 mgl^{-1} and GA_3 2.0 mgl^{-1}. C. Plantlets initiate roots in ½ MS medium containing IBA 3.0 mgl^{-1}. D. Conservation of callus in MS medium containing 2,4-D 0.2 mgl^{-1}. E. Conservation of plantlets in MS medium

CONCLUSION

From the present study it can be concluded that, in *R. Sepentina* most qualitative and quantitative callus formation is possible in half strength MS medium supplemented with 5.0 mgl^{-1} 2,4-D and 2.0 mgl^{-1} NAA. The plant multiplication was highest in full strength MS medium fortified with 6.0 mgl^{-1} BAP and 2.0 mgl^{-1} GA_3. The calli were preserved for nine months in their best condition by repeated sub-culturing in *in vitro* condition on MS medium fortified with 0.2 mgl^{-1} 2,4-D. It was also evident from the study that, the regeneration capacity of callus was reduced as the time of callusing increased.

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