IN VITRO CLONING OF AN ENDANGERED MEDICINAL PLANT, *RAUWOLFIA SERPENTINA* (L.)

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Received-29.04.2015, Revised-13.05.2015

Abstract: An efficient protocol for *in vitro* cloning of *Rauwolfia serpentina L.* was developed using leaf segment (LS), nodal segment (NS) and internodal segments (INS) as explants. The technique involves *in vitro* shoot regeneration, rooting of microshoots and transplantation of regenerated plantlets under *in vivo* condition. Sterilized explants were cultured on MS media supplemented with different auxin (IAA, IBA, NAA, & 2,4-D) and cytokinins (Kn & BAP) within a concentration range of 0.5-3.0mg/L used singly or in combination. The best shoot multiplication was obtained from nodal explants on MS medium supplemented with BAP+NAA (1.5+0.5) mg/L along with CW (5% v/v). Excellent rooting of microshoots (4-6cm) was noticed on the medium (1/2 MS salt) fortified with combination of auxins [NAA+IBA, (1+0.5)mg/L]. Compact callus which was hydrated, green and crystalline in appearance was obtained from LS and INS on medium having 1.5mg/L 2,4-D. Nodal explants were superior to internodal as well as leaf explants in response to shoot proliferation. Regenerated plantlets were transferred to pots having mixture of sand:soil:vermicuilite(1:1:1) and little fungicides (Eco fungicide). The survival rate of plantlets was much promising (around 85%) and regenerated plantlets were healthy, green and morphologically identical to mother plants.

Keywords: *Rauwolfia serpentina*, Callus, Phytohormones, Multiple shoot, Conservation

INTRODUCTION

Rauwolfia serpentina L. considered as an endangered species by IUCN (Jain *et al*. 2003) belonging to family- *Apocynaceae*, is a small, erect and glabrous shrub about 1 to 3 feet in height bearing white or pinkish flowers. It includes about 50 species having wide area of distribution including the tropical parts of the Himalayas, the Indian peninsula, Srilanka, Myanmar and Indonesia, this plant is indigenous to India, Bangladesh and other regions of Asia. The plant is commonly known as '*Sarpagandha'* and grows in wild & inaccessible places around the country (Ghani, 1998), it has restricted distribution in our locality (Muzaffarpur) and only two species of this taxon *viz; R. serpentina* and *R. tetraphylla* have been reported from Muzaffarpur (Authors). The root of this plant is of high officinal value and contains various alkaloids like; *Ajmaline, Ajmalinine, Ajmalicine, Reserpine, Scrpentine,* and *Serpentinina* (Chopra *et al*.,1956 and Srivastava *et al.*, 2006).This is an established and authentic drug plant possessing antihistaminase (Sachdev *et al.*, 1961), antihypertensive (Von Poser *et al*., 1990), sedative (Weerakoon *et al*., 1998), antiarrhythmic (Kirillova *et al*., 2001), antibacterial (Ahmed *et al*., 2002), antidiabetic (Campbell *et al*., 2006), anticancer (Bemis *et al.*, 2006), hepato-protective (Gupta *et al*., 2006), hypoglycaemic and hypolipidemic (Qureshi *et al*., 2009), anti inflammatory (Rao *et al*., 2012) and antidiarrhoeal (Ezeigbo *et al*., 2012) activities in addition to marked hypnotic & sedative properties, that's why it is used in the treatment of epilepsy, sleeplessness, hypertension and other ailments (Joshi & Kumar, 2000 and Manuchair, 2002).

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The seed germination percentage is very low upto 20% due to presence of cinnamic acid derivatives (Mitra, 1976; Paul *et al.* 2008; Sushila *et al*. 2013). Poor seed viability, low seed germination rate and enormous genetic variability are the major constraints for the commercial cultivation of *R. serpentina* through conventional methods. The increasing demand for *Rauwolfia* roots in national and international markets and decreasing availability have encouraged many farmers to cultivate this pharmaceutically important plants on large scale, Government of Bihar has been encouraging farmers for cultivation of some rare medicinal plants including *Rauwolfia serpentina* by way of providing subsidies (AAP, 2009-10). Uniform and mass propagation through *in vitro* method would be beneficial for the germplasm conservation, systematic cultivation and commercial exploitation of this endangered species for the production of reserpine and other active constituents at desirable level. Keeping these into consideration, the present investigation was undertaken to develop a simple and efficient protocol for *in vitro* cloning on large scale using leaf, node & internode segments as explants.

MATERIALS AND METHOD

Explants [Nodal segment(NS), internodal segment(INS) & leaf segment(LS)] collected from 9-12 month old *in vivo* grown *Rauwolfia* plant (from Campus of B R A Bihar University, Muzaffarpur, Fig.1) were washed thoroughly under running tap water for 15-20 minutes and then treated with 1% Savlon along with 4-5 drops of Tween80 for about 20-25 minutes with constant shaking followed by 3-4

times washing with double distilled water (DDW) to make the material free from detergents. Explants were immersed in 0.1% $HgCl_2$ for 3-5 minutes and finally rinsed with sterilized DDW.

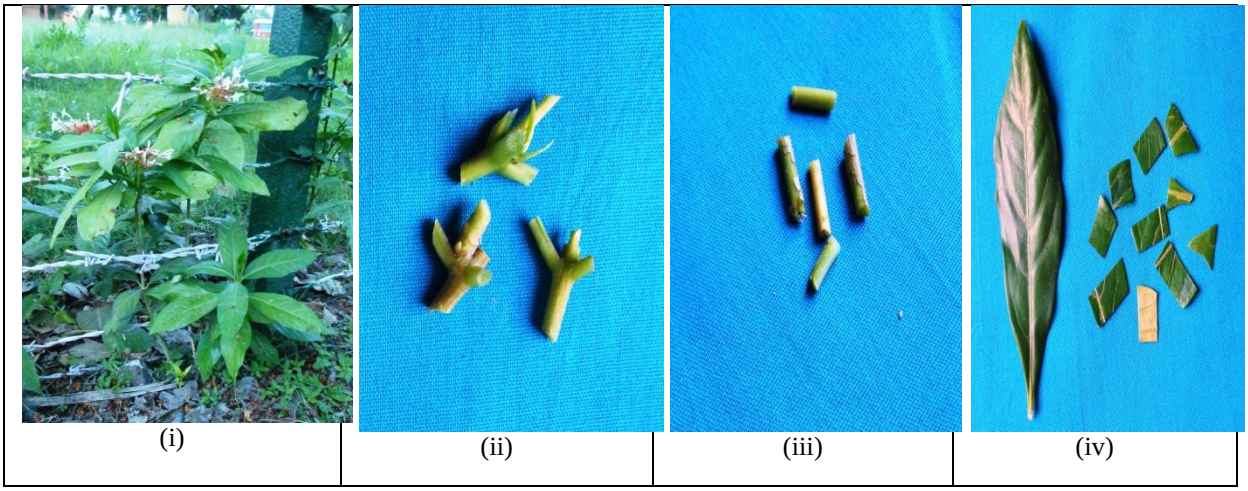

Fig.1: (i)12 Months old *in vivo R.serpentina* plant (ii) Nodal explants/NS (iii) Internodal explants/INS (iv)Leaf explants/LS

Sterilized explants (NS, 8-12mm, INS, 8-12mm, LS-6x8mm) were aseptically cut and inoculated singly in culture tubes (25x150mm) containing MS (Murashige & Skoog, 1962) medium with 3% sucrose 0.8% agar and different combination and concentration of auxin and cytokinin (Table- 1, 2 & 3). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 minutes. Cultures were maintained at 25 ± 2°C with light intensity of 2000 lux (Cool, white) under 16/8 hour photo cycle.

Ten replicates were maintained for each experiment and were repeated twice. Shoot proliferation and elongation lasted for 6-7 weeks, callus induction lasted 5-6 weeks and rooting for 4-5 weeks, the time of each stage was fixed. Percentages of explants showing shoot proliferation, number of shoots/explants and lengths of shoots were taken as parameters for evaluating the morphogenic potentialities of explants in the present experimental system. Calli were maintained for a long term (about 2 years) by sub-culturing every 4-5 weeks on suitable medium.

RESULTS AND DISCUSSION

Callus induction

Callus started to form on LS and INS explants from cut ends after 10 and 14 days of inoculation respectively on MS media having different combination and concentration of hormones

Table 1. Callus induction from leaf and internodal segments of *Rauwolfia serpentina* under effective concentration and combination of phytohormones (30 Days old culture)*

Hormonal concentration(mg/L)				*Biomass of Callus (in mg)*		*% of responding explants*		*Nature of Callus*		*Other response*
IBA	*NAA*	*2,4-D*	*BAP*	*LS*	*INS*	*LS*	*INS*	*LS*	*INS*	
0.5	--	--	--	206±12.60	201±13.7	35±4.0	28±2.0	WF	WF	--
1.5	--	--	--	465±30.14	407±22.2	60±4.4	55±2.2	GC	WF	--
2.5	--	--	--	071±06.00	065±04.2	27±4.4	22±4.8	BC	BF	--
--	0.5	--	0.5	535±13.40	507±16.4	52±4.1	50±3.7	WC	WC	--
--	0.5	--	1.5	426±20.60	415±19.3	45±3.0	42±3.2	GC	WC	**
--	0.5	--	2.5	290±22.90	250±27.2	38±3.5	32±2.4	HG	WF	--
--	--	0.5	--	490±20.30	478±18.6	65±5.6	60±4.3	HG	WC	--
--	--	1.5	--	687±25.10	603±26.5	82±4.2	80±4.5	HG	WC	--
--	--	2.5	--	06±18.20	501±38.1	55±3.2	53±6.2	HG	WC	--
--	--	1.5	0.5	472±15.60	460±17.1	51±5.6	46±3.6	WC	BC	--
--	--	2.5	0.5	236±09.60	157±10.3	37±2.7	30±3.6	WC	WF	--

*Data scored (Mean ± SE) from 10 replicates of LS and INS explants which were repeated twice.

** Shoot buds emergence

Table 2. Direct shoot regeneration from nodal explants of *Rauwolfia serpentina* on effective concentration and combination of growth regulators (28 Days old culture)*

Hormonal concentration (mg/L)						
Kn	***BAP***	***NAA***	***IBA***	**CW(v/v)**	***Total no. of shoots***	***Mean length of shoot(cm)***
0.5	0.0	0.0	0.0	0%	1.19±0.23	0.11±0.19
1.5	0.0	0.0	0.0	0%	2.10±0.27	0.58±0.26
2.5	0.0	0.0	0.0	0%	1.65±0.16	1.15±0.15
0.0	0.5	0.0	0.0	0%	2.20±0.34	1.70±0.10
0.0	1.5	0.0	0.0	0%	3.89±0.12	4.20±0.20
0.0	2.5	0.0	0.0	0%	3.35±0.28	2.90±0.34
0.0	3.0	0.0	0.0	0%	1.91±0.15	2.12±0.14
0.0	1.5	0.5	0.0	0%	3.92±0.54	4.60±0.49
0.0	1.5	0.5	0.0	05%	5.20±0.42	6.50±0.15
0.0	1.5	0.5	0.0	10%	4.30±0.42	4.95±0.40
0.0	1.5	1.0	0.0	0%	2.95±0.19	3.01±0.23
0.0	2.5	2.0	0.0	0%	1.54±0.34	1.90±030
0.0	1.5	0.0	0.5	0%	3.40±0.12	3.80±0.45
0.0	2.5	0.0	1.5	0%	1.50±0.12	1.30±0.11

*Data scored (Mean ± SE) from 10 replicates of LS and INS explants which were repeated twice.

Table 3. Rooting of micro-shoots of *Rauwolfia serpentina* on media having effective concentration and combination of phytohprmones (25 days old cultures)*

[Hormonal concentration (mg)/L]					
Medium	***NAA***	***IBA***	***IAA***	***Total no. of roots/culture***	***% of response/culture***
MS	0.5-2.0	0.5-2.0	0.0	--	--
	0.5-3.0	0.5-3.0	0.0	--	--
½ MS	0.5	0.0	0.0	2.9±0.11	52±2.81
	1.0	0.0	0.0	3.5±0.15	60±4.30
	1.5	0.0	0.0	2.4±0.12	47±3.52
	0.0	0.5	0.0	5.3±0.14	70±2.40
	0.0	1.0	0.0	3.8±0.13	66±2.10
	0.0	1.5	0.0	2.6±0.15	56±2.23
	0.0	0.5	0.5	1.8±0.28	46±2.27
	0.0	1.0	0.5	2.5±0.21	49±3.20
	0.0	1.5	0.5	1.3±0.17	30±2.11
	0.0	0.0	0.0	2.0±0.24	42±2.80
	0.0	0.0	0.0	1.6±0.32	35±3.32
	0.5	0.5	0.0	3.8±0.20	65±2.23
	1.0	0.5	0.0	6.2±0.25	81±3.92
	1.5	0.5	0.0	3.3±0.12	60±4.49

*Data scored (Mean ± SE) from 10 replicates of microshoots which were repeated twice.

[LS- leaf segment, **INS**- Inter nodal segment, **NS**- Nodal segment, **IAA**- Indol-3- acetic acid, **NAA**- naphthalene acetic acid, **Kn**- Kinetine**,** **IBA**- Indole butyric acid, **2,4-D**- 2,4-dichlorophenoxyacetic acid, **BAP**- 6-benzlamonopurine, **WC**- white crystalline, **HG**- hydrated green, **BC**- brown compact, **WF**- white friable, **BF**- brown friable**,** **GC**- Green compact.]

(Table 1), the best callus biomass both in LS (687±25.10)mg and INS (603±26.5)mg was obtained on 2,4-D (1.5mg/L, Fig. A & B) and the % response of explants was also optimum on the above combination of hormone (Table 1). Callogenesis in explants (LS, INS) was encountered in all the combination of hormones tested (Table 1), promising results were also noted on BAP+NAA (0.5+0.5, 1.5+0.5)mg/L in addition to 2,4-D (Table 1). Callus induction in NS was not encouraging on any combination of hormones and hormones above 3 mg/L had adverse effect on callus initiation and explants finally turned brown. These findings are congruent with the observations of Patil &Jayanti (1997) and Singh *et al*. (2009). The callus in general was crystalline, hydrated, green and compact in texture, however, calli were white & friable in some combination of hormones (Table 1).

A -*In vitro* callus initiation from INS-explants on the medium MS+1.5mg/L 2,4-D (16 days old)
B -Callus induction from LS explants on the medium having MS+(1.5)mg/L 2,4-D (20 days old)
C-Callus mediated multiple shoots on medium MS+(1.5+0.5)mg/L BAP+NAA on subculture(20 days old)
D -Growth of isolated callus mediated shoots on medium having 0.5mg/L Kn (15 days old)
E-Shoot regeneration from NS on medium supplemented with BAP+NAA (1.5+0.5)mg/L added with CW 5%v/v (28 days old)
F-35 Days old *in vitro* shoots bearing pink flower bud on the above medium mentioned in fig. E
G & **H**- Successful rooting of microshoots on medium fortified with ½ MS+(1+0.5)mg/L NAA+IBA (25 days old)
I- Hardening of plantlets in the plastic pots having sand+soil+vermiculites(1:1:1), 30 days old acclimatized *in vitro* raised *Rauwolfia* plants.

2,4-D is usually the choice of auxin for callus induction in the present experiment system but 2,4-D alone or in combination with BAP was not suitable for long term culture and callus mediated regeneration, as also reported by Bhaskaran & Smith (1990), Naseem & Jha (1994) and Chaudhury & Qu (2000). The callus was conserved for a period of about 2 years by regular sub-culturing at interval of 30-35 days on MS medium supplemented with BAP+NAA (0.5+0.5)mg/L. Callus induction was limited by several factors, when these requirements were adequate (temperature 25±2°C, pH- 5.8 and 2000 lux light), the culture response was maximum.

Callus mediated shoot regeneration

Green calli were only potent for shoot regeneration on subculture medium (MS) containing NAA and BAP within a concentration range of 0.5-3.0mg/L, the best shoot regeneration from callus in bunch was obtained on BAP+NAA (1.5+0.5)mg/L (Fig. C) after 15 days of subculture and percentage response on this combination was also much promising (about 81%). Non-green calli could not respond to the medium and showed complete loss of differentiation and regeneration even on different media and hormonal combinations, this was also evidenced by Narayanswamy (1977) and Naseem & Jha (1994). Tiny shoots (about 2cm) were isolated and sub-cultured on MS medium containing only 0.5mg/L Kn for shoot elongation (Fig. D). From the present findings, it is evident that cytokinin alone as well as high cytokinin & low auxin ratio promotes shoot regeneration and elongation (Singh *et al.* 2009, Kumar *et al*.2010, Mallick *et al.* 2012 and Sushila *et al*. 2013).

Shoot regeneration

Direct regeneration of shoots was obtained from nodal explants supported with different auxin (NAA & IBA) and cytokinin (BAP& Kn) in various combination and concentration within a range of 0.5-3.0mg/L (Table 2), multiple shoot induction as well as optimum shoot length was recorded on medium supplemented with BAP+NAA (1.5+0.5)mg/L addendum with 5% v/v CW(Fig. E). Bulging and hypertrophy in nodal explants was prominent before initiation of shoot buds. Regeneration of shoots on suitable medium was noticed after 10 days of inoculation. Table 2 shows that promising number of shoots was also obtained on MS medium containing BAP+NAA (1.5+0.5)mg/L along with
10% v/v CW and BAP+NAA (1.5+0.5)mg/L without CW. Growth regulators [BAP+NAA (1.5+0.5)mg/L] and CW (5%v/v) together exhibited synergistic effect and induced better shoot regeneration (Table 2). Such an effect of CW and growth hormone was also recorded by Mukhopadhayay & Sharma (1986) and Naseem & Jha (1994). Direct shoot bud regeneration in the present system depends on quantitative interaction of auxin and cytokinin, this was also reported in *Delbergia lenceoleria* (Dwari & Chand, 1996) and *Aloe vera* (Khanam *et al,* 2014). Tissue culture studies on a number of medicinal plants including *R. serpentina* (Naseem & Jha, 1997, Singh *et al*. 2009, Kumar *et al*. 2010, Mallick *et al.* 2012 and Khanam *et al.* 2014) suggest that a fine balance of exogenous auxin and cytokinin is necessary before successful regeneration can occur, hormones above 3 mg/L and CW above 10% v/v had adverse effect on shoot multiplication. *In vitro* florigenesis and optimum shoot elongation (6.5±0.15) cm were recorded after 28 days of culture on suitable combination of hormones (Table 2, Fig. F). Florigenesis in culture, an event of biological interest was also reported by Tran Tanh van (1973), Naseem &Jha (1994) and Patil & Jayanti (1997).

In vitro propagation in *Rauwolfia* from auxillary buds has proved to be the most acceptable and reliable method (Mallick *et al.* 2012; Sushila *et al.* 2013) as the regeneration of plants from seed source is difficult. Percentage seed germination in this plant is not encouraging as reported by Dutta *et al* (1962) and Paul *et al*. (2008), nodal explants as a means of micropropagation have been reported in a number of texa including *R.serpentina* as an ideal explant for direct shoot multiplication (Salma *et al.* 2008; Kumar *et al.* 2010 and Mallick *et al*. 2012). Direct regeneration of multiple shoots/ shoots from nodal culture is highly desirable since the regenerants are genetically identical to the mother plant (Naseem and Jha, 1997 and Mallick *et al.* 2012).

Rooting and plantlet formation

Regenerated microshoots (4-6cm) obtained in 3.2 and 3.3 were excised and individually implanted on MS as well as rooting media *i.e* ½ MS+PGR (Plant growth regulator) for rhizogenesis, rooting was obtained on ½ MS medium in presence of different auxin (IAA, NAA and IBA) either used singly or in combination within a concentration range of 0.5-2.0mg/L and optimum rooting of microshoots (Fig. G & H) was achieved on ½ MS supplemented with NAA+IBA(1+0.5)mg/L within 20 days. Low salt medium with combination of auxin (NAA+IBA) have been found to have stimulatory effect on root induction in many plant species (Laxmi-Sita *et al*. 1986 and Naseem & Jha, 1994). No rhizogenesis was recorded on MS medium with/without PGR (Table 3). Physical growth conditions described under callus induction were also optimal for rhizogenesis. Regenerated plantlets were transferred to plastic pots (Fig. I) having sterilised soil mixture (vermiculites+sand+soil, 1:1:1) and little fungicide (Eco fungicide), plantlets were acclimatized for a week in culture room and finally transferred to shade house. *In vitro* raised plantlets were healthy, green and morphologically identical to mother plants and the survival rate of plantlets was also encouraging (around 85%).

CONCLUSION

In the present experiment, multiple shoots/shoots developed directly [NS] and indirectly *via* Callus formation [INS, LS] can be used as ideal explants for *in vitro* cloninig of *R. Serpentina* and mass propagation achieved by this method is highly efficient & productive.

ACKNOWLEDGEMENT

Authors are grateful to University grant commission (UGC), New Delhi for providing fellowship as financial support and Professor and Head of University Department of Botany, B R A University, Muzaffarpur for all other required necessities related to this experiment.

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