www.bti.org.in
ISSN 0974-1453
Research Article

IN VITRO REGENERATION OF *RAUWOLFIA SERPENTINE* THROUGH ANTHER CULTURE STUDIES

Varnika Chaudhary[1], Shalini Singh[1], Sandhya Vaid[2], Nishesh sharma[2*]

[1]**Department of Biotechnology, Dolphin (PG) Institute of Biomedical and Natural Science, Dehradun.**
[2]**Department of Biotechnology& Zoology, Chinmaya Degree College, Haridwar.**
***Corresponding author: nishesh21@gmail.com**

ABSTRACT

***Rauwolfia serpentina* is an important medicinal plant which has become endangered mainly due to over exploitation from natural stands. Hence there is a need to develop methods and strategies for conservation of the plant. Present study provide an easy, simple and efficient method of development of extensive callusing from in vitro culture of anthers of *Rauwolfia serpentina*.MS medium fortified with different concentration of Kn, NAA or 2,4-D were all find effective enough to induce callus development from cultured anthers of *Rauwolfia serpentina*.**

Keywords: Anther, *in vitro*, callusing, regeneration.

INTRODUCTION

Rauwolfia serpentine (L.) Benth is an important medicinal plant belonging to family Apocynaceae. The plant is commonly found in India, Nepal, Bangladesh, Pakistan, Srilanka, Burma and Thailand (Roy *et al.*, 1994; Dey and De, 2010). The plant is an evergreen shrub which has been utilized for treatment of various diseases such as high blood pressure, nervous system disorders, anxiety, epilepsy, insanity, schizophrenia (Dastur, 1998; Kirtikar and Basu,1993; Bhatara *et al*, 1997) and roots of plant are specifically used against Cholera, Diarrhea, Dysentery (Ghani, 1998, Tona *et al.*, 1999) *R. serpentine* is better known as sarpgandha or snake root plant since the plant is extremely effective against snake bite, stings of insects and other poisonous reptiles (Bhatt *et al.*, 2008). Plant is known to possess several bioactive metabolites which are responsible for the immense medicinal potential of the plant. Serpentine, reserpine, deserpidine, ajmaline are few of the important metabolites (Achmad, 1987). *Rauvolfia serpentine* threatened with extinction in India mainly due to unrestricted collection from natural sources for traditional purposes and to fulfill the demand from pharmaceutical sector (Nayar and Sastry, 1987, 1988, 1990; Mamgain *et*

al., 1998; Singh *et al.,* 2010). Several researchers have confirmed the endangered status of the plant (Sethi and Kazim, 1983, Nayar, 1996, Jadhav *et al,* 2001, Raj and Sukumaran, 2008, Mao *et al,* 2009). Poor seed viability along with low germination rate have also contributed to present endangered status of the plant. Presence of cinnamic acid seed is considered to be responsible for low germination rate (Mitra, 1976; Sahu, 1979). Vegetative propagation has proved to be insufficient to meet the demands of the plant (Salma *et al.,* 2008). Due to the prevailing reasons there is a huge need for conservation, mass propagation and sustainable utilization of *R. serpentine.* Hence improvements in plant tissue culture techniques for the mass propagation of *R. serpentine* are highly desirable. Tissue culture studies avail a suitable alternative for conservation of the plant. Several studies have been conducted utilizing nodes and leaves of the plant as explants (Jain *et al.,* 2003, Bhatt *et al.,* 2008, Nurcahyani *et al.,* 2008, Bahuguna *et al.,* 2011). Present study depicts an easy and much more efficient method of mass propagation of *R. serpentine* through in vitro culture of anthers.

MATERIALS AND METHODS

Young plants of *Rauwolfia serpentine* were procured from Botanical garden of Patanjali Yogpith, Haridwar and were maintained in green house for further growth. Anthers obtained from mature plants were utilized as explants in the present study.

Anthers were gently washed with tap water for 4-5 min and were treated with 1% bavastein solution for not more than 2 min following which anthers were thoroughly washed with tap water to remove all traces of bavastein. Anthers were then transferred to LAF for further sterilization where they were surface sterilized with 0.1% $HgCl_2$ for 1-2 min. Anthers were then washed 4-5 times with sterile distilled water and dried using autoclaved tissue paper before inoculation. MS medium was utilized as basal medium for the present study (Murasige and Skoog, 1962). Separate experiments were conducted to evaluate effectiveness of PGR's Kn, NAA, and 2,4-D. Different media combinations containing varying concentration of these hormones were utilized test medium for the present study.

Cultures were regularly monitored for morphogenic response and observations made were tabulated as % culture response, nature and extent of callus obtained, average and maximum number of shoots regenerated from callus. Each experiment consisted of minimum 20 cultures and was repeated at least three and mean value was recorded and tabulated.

RESULTS AND DISCUSSION

When surface sterilized anthers were cultured on to MS medium containing 4µM Kn about 48.6% cultures exhibited callus induction within 10-12 days of inoculation. The extent of callusing was moderate onto this concentration. Callus obtained was compact in nature and cream to brown in colour (Fig. 2A) which eventually turned brown on prolonged culture (Fig. 2B). Callus development initially began from lower part of anther and some of the anthers also exhibited flowering along with callus development. When the concentration of Kn was increases upto 8-10µM. Percentage of

cultures developing callus was accordingly enhanced to 70-84.6% respectively. Elaborate callus development was observed onto these concentrations. However, on further increasing concentration of Kn (12-14µM), percentage of cultures developing callus was comparatively low (about 74.2-68.0) as compared to previous concentrations of Kn (Table1). Also, moderate callus was obtained onto 14µM Kn. Irrespective of the response obtained on different concentration of Kn cultured anthers initially exhibited swelling and enlargement in size before callusogenesis.

Similar kind of results were also obtained onto medium fortified with different concentration of NAA (4-12µM) or 2,4-D (2-14µM) in independent experiments. Several studies conducted on numerous plant species have revealed the effectiveness of both the auxins, NAA and 2,4-D in inducing callus from different explants. Both the hormones exhibit equal potential to induce morphogenic response. However, the kind of response obtained may vary depending upon the plant species and also on the explants selected. In the present study response of 2,4-D on anther culture study was found to be far superior as compared to NAA in terms of % cultures exhibiting callus development and extent of callus obtained.

Onto medium fortified with 4µM NAA about 48.0% cultures developed callus within 15-20 days of culture. A maximum of 68.4-72.6 cultures developed callus when concentration of NAA was increased upto 8-10µM respectively. Callus obtained was creamish, fragile and moderate in extent on all concentrations of NAA. Onto MS+4µM 2,4-D about 58.6% cultures developed callus. On increasing concentration of 2,4-D to 8µM about 69.4% cultures developed callus. Elaborate extent of callusing was achieved onto 4-8µM 2,4-D as compared to same concentration of NAA (Fig. 3A-C). A maximum of 82.5% cultures exhibited callusing onto medium composition, MS+12µM 2, 4-D (Table 1). Callus obtained was creamish and compact onto this concentration. Extensive callus growth was achieved. As seen in results obtained onto Kn supplemented medium swelling and enlargement in size of anther before callus induction was also achieved onto medium containing either 2,4-D or NAA (irrespective of concentration). Medium supplemented with Kn, NAA or 2,4-D have resulted in appreciable morphogenic response in terms of callus development from cultured anthers of *R. serpentine*.

In most of the previous studies conducted pertaining to callus induction (from node, leaf segments) at least a combination of two hormones have been utilized (Bhatt *et al.,* 2008, Nurechyani *et al.,* 2008, Bahuguna *et al.,* 2011). Contrary to that in the present study sufficient amount of callus induction was obtained by culturing anther onto medium fortified with single PGR (Kn or NAA or 2,4-D). Although MS +Kn , MS + 2,4-D and MS + NAA have resulted in appreciable amount of callus induction, however callus obtained onto all these hormonal media combinations failed to regenerate further unless sub-cultured. Among various media compositions utilized for regeneration of shoot buds from callus, MS + BAP + IBA was found to be most effective. Onto MS+

BAP (8-10 µM) + IBA (4-10 µM) all the cultures exhibited regeneration of shoot buds from regenerated callus (Table 2, Fig.4A-C). Similar kind of regeneration from callus derived from leaf and stem segments have also been reported by Panwar *et al.,* 2011. However, in this study we also achieved regeneration onto MS + BAP and MS+BAP + NAA, beside MS +BAP + IBA.

Elongated shoots were transferred to freshly prepared rooting medium, MS+ IBA (5-25 µM) for induction of root formation from in vitro regenerated shoots. About 28.6 –44.8% cultures developed roots when concentration of IBA was 5-10 µM. A maximum of 78.6% cultures developed roots onto MS+ 20 µM IBA with an average of 7.3 ± 1.2 roots per explants (Table 3, Fig. 5A). The results obtained are in concordance with findings of Jain *et al.,* (2003) and Panwar *et al.,* (2011). However, Bahuguna *et al.,* (2011) in their study found MS+IBA +IAA to be most appropriate medium for *in vitro* rooting.

Conclusion

By far this is one of the initial reports pertaining to tissue culture study of anthers of *R. serpentina*. The study provides an easy method involving single hormone treatment for extensive callus growth. The protocol can further be utilized to analyze biochemical and molecular changes occurring during callogenesis and variation in extent of metabolites produced.

Table 1: Response of Anther culture to different PGR's

PGR's	**Swelling**	**% Culture developing callus**	**Nature of callus**	**Degree of callusing**
Kn				
4	Y	48.6	Creamish brown / compact	++
8	Y	70	Creamish brown / compact	+++
10	Y	84.6	Creamish brown / compact	+++
12	Y	74.4	Creamish brown / compact	+++
14	Y	70.2	Creamish brown / compact	++
NAA	**Swelling**	**% Culture developing callus**	**Nature of callus**	**Degree of callusing**
4	Y	48	Creamish white / fragile	++
8	Y	68.4	Creamish white / fragile	++
10	Y	72.6	Creamish white / fragile	+++
12	Y	78.5	Creamish white / fragile	++
2,4-D	**Swelling**	**% Culture developing callus**	**Nature of callus**	**Degree of callusing**

4	Y	58.6	Creamish white /compact	++
8	Y	69	Creamish white /compact	++++
12	Y	82.5	Creamish white / fragile	++++
14	Y	78.4	Creamish white / fragile	++++
16	Y	76.5	Creamish white / fragile	++++

++ (Moderate degree of callusing), +++ (good degree of callusing), ++++ (extensive callusing);
Y= swelling occurs, N= no swelling.

Table 2: Regeneration of shoot buds from callus

BAP	IBA	% culture exhibiting regeneration	Max. No. of shoots
2	-	N	N
4	-	N	N
8	-	N	N
12	-	N	N
-	2	N	N
-	4	N	N
-	8	N	N
-	12	N	N
2	4	21.3	4
4	4	26.4	6
4	8	38.2	5
8	4	100	8
8	8	100	13
10	4	100	11
10	10	100	14

N = no response/ growth

Table 3: *In vitro* rooting from regenerated shoots

IBA	% culture developing roots	Avg. no. of roots	Avg. root length (cm)
5	28.6	4.4±0.3	5.2
10	44.8	6.1±0.7	4.3
15	48.2	6.3±0.5	7.4
20	8.6	7.3±1.2	10.2
25	72	6.0±0.6	9.6

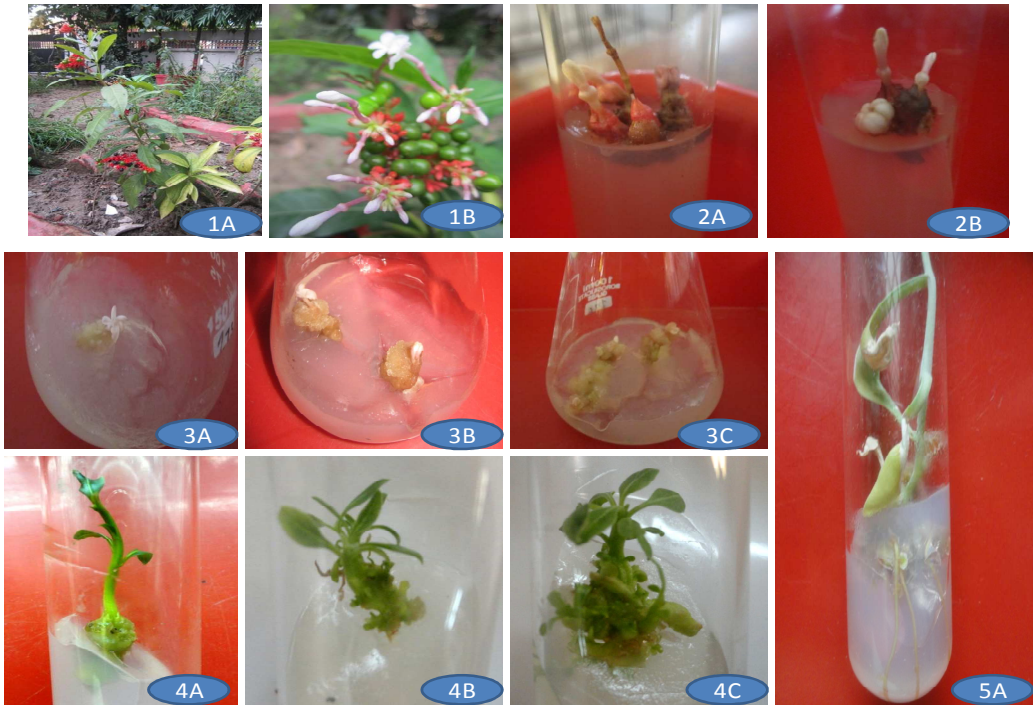

Figure 1(A-B). Mature plants of *R. serpentina* exhibiting flowering, **2(A-B).** callus development from anthers onto MS+4µM Kn, **3A.** Callusing onto MS+8µM NAA, **3B**. Callusing onto MS+8µM 2,4-D, **4(A-C).** Regeneration from callus onto MS+BAP+IBA, **5A.** *In vitro* rooting onto MS+IBA.

REFERENCES

Achmad, S.A. (1987). Metabolit sekunder: suatu kerangka untuk memahamipotensi sumber daya alam nabati Indonesia. *Dalam:* Pramono, S., D.Gunawan, dan C.J. Soegihardjo. (ed.). *Buku Risalah Seminar Nasional Metabolit Sekunder*. Yogyakarta: PAU Bioteknologi UGM.

Bahuguna, R.N., R. Joshi., G. Singh., A. Shukla., R. Gupta., G. Bains (2011). Micropropagation antotal alkaloid extraction of Indian snake root(*Rauwolfia serpentina*): an important antihypersensitive medicinal shrub. Indian J. AgricRes., 81: 1124-1129.

Bhatara,V.S., Sharma J.S., S.Gupta., Y.K.Gupta, (1997). Images in Psychiatry. *Rauwolfia serpentine*: The first herbal antipsychotic.Am.J.Psychiatry,154 (7):894.

Bhatt, R., Ariff, M., Gaur, A.K., Rao, P.G. (2008). *Rauwolfia serpentina*: Protocol optimization for *in vitro* propagation. Afr. J. Biotech. 7:42-65.

Dastur, J.F. (1998). Medicinal plants of India and Pakistan. D.B Taraporevala and sons, New Delhi:141-142.

Dey, A and De, J.N. (2010). Rauwolfia serpentina(L.) Benth.ex. Kurz.-A

Review, Asian Journal of Plant Sciences 9: 285-298.

Ghani, A. (1998). Medicinal plants of Bangladesh. Chemical constituents and uses. Asiatic Society of Bangladesh, Ed. 2nd pp. 36.

Jadhav,S.N., K.N. Reddy., Reddy, C.S. (2001). Conservation assessment and management planning for medicinal plants of Andhra Pradesh. Medicinal plants conservation center (MPCC) and FRLHT, Hyderabad, India, pp:1-39.

Jain, S.P., Singh, J., Singh, S.C. (2003). Rare and endangered medicinal and aromatic plants of Madhya Pradesh. J. Economic and Taxonomic of Botany, 27: 925-932.

Kirtikar, K.R. and Basu, B.D. (1993). Indian medicinal plants. vol.2. DehraDun Publishers, Culcutta,India.289.

Mamgain, S.K., Goel, A.K., Sharma, S.C. (1998). Conservation of assessment of some important threatened medicinal plants of India. J. Non-Timber Fore. Prod. 5: 1-9.

Mitra, G. C. (1976). Studies on the formation of viable and non-viable seeds in *Rauwolfia serpentina* benth. Indian J. Exp. Biol. 14: 54-56.

Moa, A.A., Hynniewta, T.M., Sanjappa, M. (2009). Plant Wealth of northeast India with reference to ethnobotany. Ind. J. Trad. Knowledge. 8:96-103.

Murashige, T.S., Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-479.

Nayar, M.P and Sastry, A.R.K. (1987). Red Data Book of Indian Plants. Vol. 1, Botanical Survey of India, Calcutta.

Nayar, M.P. and Sastry, A.R.K. (1990). Red Data Book of Indian Plants. Vol. 3, Botanical Survey of India, Calcutta.

Nayar, M.P. and Sastry, A.R.K. (1988). Red Data Book of Indian Plants. Vol. 2, Botanical Survey of India, Calcutta.

Nayar, M.P. (1996). Hot spots of Endemic Plants in India, Nepal and Bhutan. Tropical Botanical Garden and Research Institute, Kerala, pp:252.Thiruvananthapuram.

Panwar, G. S., Attitalla, I. H. and Guru, S. K.(2011). An efficient in vitro clonal propagation and estimation of reserpine content in different parts of *Rauwolfia serpentina* L., American-Eurasian Journal of Scientific Research 6:217-222.

Raj, A.D.S. and Sukumaran, S. (2008). Rare and endemic plants in the sacred groves of Akumari district in Tamil Nadu. Ind. J. Forestry, 31:611-616.

Ramawat, K.G. (1999). Production in culture: optimization. *In:* Ramawat, K.G.and J.M. Merillon. (eds.) *Biotechnology Secondary Metabolites*. NewHampshire: Science Publishers, Inc. *of Biological Sciences* 11: 1638–41.

Roy, S.K., Hossain, M.Z., Islam, M.S. (1994) Mass Propagation of *Rauvolfia serpentina* by *in vitro* Shoot Tip Culture. Plant Tissue Cult. 4:69-75.

Sahu, B.N. (1979). *Rauwolfia*: Botany and Agronomy, Vol. 1, Today and Tomorrow's Printers and Publishers, New Delhi, India.

Salma, U., M.S.M. Rahman, S. Islam, N. Haque, T.A. Jubair, A.K.M.F. Haque and I.J. Mukti (2008). The influence of different hormone concentration and combination on callus induction and regeneration of *Rauwolfia serpentina* L. Benth. Pakistan Journal of Bioscience. *11: 1638-1641.*

Sethi., K.L. and Kazim, M. (1983). Collection and Conservation of Rauwolfia serpentina genetic resources of south Karnataka and Western Ghats of India. Proceeding of the 5th All India workshop on Medicinal and Aromatic Plants, Oct.83,Solan, Indian.1-8.

Singh, P.K., Kumar, V., Tiwari, R.K., Sharma, A., Rao, C.V. and Singh, R.H. (2010). Medico-ethnobotany of Chatara block of district Sonebhadra, Uttar Pradesh,India. Adv.Biol. Res.4:65-80.

Tona,L,K.,Kambu,K.,Mesia,K.,Cimanga,S., Apers,T.,De Bruyne. L., Pieters, J.,Totte., Vlietinck, A.J. (1999). Biological screening of traditional preparations from some medicinal plants used as antidiarrhoeal in Kinshasa,Congo.Phytomedicine.6: 59-66.