



DIRECT *IN VITRO* REGENERATION FROM LEAF SEGMENTS OF *RAUWOLFIA SERPENTINE*

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ABSTRACT: *Rauwolfia serpentina* commonly known as Sarp Gandha is a well known medicinal plant possessing several medicinal and pharmacological activities. Extreme effectiveness of plant against snake and insect bites have made the plant popular around the globe. Over the time plant has become endangered mainly due to overexploitation and comparatively moderate propagation rate in nature. Present study reports a method of direct *in vitro* regeneration of shoot from *in vitro* cultured leaf segments of *R. serpentina*. About 68% cultured developed *in vitro* shoots from cultured leaf segments onto MS+8 μ M NAA+4 μ M BAP. Medium enriched with higher concentration of NAA was also found suitable for *in vitro* rooting.

Key words: *In vitro* culture, leaf, conservation, *Rauwolfia*

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INTRODUCTION

Rauwolfia serpentina (Sarp Gandha) belonging to family Apocynaceae is an important medicinal plant. The plant is commonly distributed in foothills of Himalayan region and is found in India, China, Nepal, Sri Lanka, Burma, Thailand [1,2]. The plant has been reported to be effective in several diseases and disorder such as diarrhea, dysentery, insomnia, insanity etc. However, the most common utilization of the plant remains against the stings and bites of insects and poisonous snakes and many other reptiles [3]. The medicinal importance of plant is attributed to presence of several alkaloids among which reserpine is the most important [4].

In the recent past, the abundance of plants of *R. serpentina* have greatly declined from natural habitat due to over exploitation, poor seed germination and destruction of habitat [2,5,6,7,8]. The plant has eventually been listed as endangered by International Union for the Conservation of Nature and Natural Resources (IUCN) [9]. Hence, there is a need to develop protocol for mass propagation and conservation of the plant. Plant tissue culture is a well known technique which has been successfully utilized for mass propagation of several plant species. Generally, segments form the most suitable explant for tissue culture studies but since the plant do not exhibit branching and hence utilization of nodes as explant results in cutting of a well grown mature plant. But if instead of nodes leaves are used as explant then the mother plant will also remain intact and several other plants can be generated *in vitro*.

Hence, the present study reports direct shoot regeneration from *in vitro* cultured leaves of *R. serpentina*.

MATERIAL AND METHODS

Plants of *R. serpentine* were procured from botanical garden of Patanjali Yogpeeth and maintained in Department of Biotechnology, Chinmaya Degree College, Haridwar. Young leaves from these plants (Fig. 1A) were utilized as explant in the present study.

Leaves were thoroughly washed with tap water for about 5 minutes followed by treatment with tween 20 for further 5 minutes. Leaves were again washed to remove all traces of tween 20. Leaves were washed with distilled water and revised with 70% ethanol for about 30 seconds. Explant were again washed with sterile distilled water and transferred to laminar airflow. Leaves were finally surface sterilized with 0.1% HgCl₂ for 2-3 minutes and then washed with sterile distilled water 3-4 times, leaves were then dried using sterile tissue paper and excised into segment, and then inoculated onto prepared medium.

MS medium [10] was utilized as basal medium for the present study. Media supplemented with different auxin 2,4-D and NAA either alone or in combination with cytokinin BAP in varying concentration (2-12µM) in separate experiments were utilized as culture medium to evaluate response of *in vitro* cultured leaf segments. Regenerated shoot were aseptically excised and transferred to either basal medium or medium containing same hormone on which shoots were regenerated for further elongation if required. Well elongated shoots were transferred to freshly prepared rooting medium for *in vitro* root induction.

Full as well as half MS medium supplemented with plant growth regulators (IAA, IBA, NAA) in varying concentration (5-20µM) was utilized for *in vitro* root induction. Well developed plants were acclimatized. Plants were removed from test tube under aseptic conditions and washed gently with sterile water to remove all traces of media. Then they were planted in pots containing sterile sand and soil in ratio 1:1. Pots were covered with transparent poly bags and regularly watered with ½ strength major solution. Poly bags were cut from corner after 7-8 days of plant survival and were completely removed after 15-20 days. Plants were then maintained in green house and were eventually transferred to natural soil.

All the chemicals used for preparation of medium were obtained from CDH, New Delhi India. The cultures were incubated at 26 ± 20°C under 16hrs light photoperiod of 15µE/m² /s irradiance provided by Philips fluorescent tubes (36W/54, 6500K) in the culture room. For each treatment, a minimum of 20 cultures were raised and each experiment was repeated at least twice. The cultures were examined periodically and the morphological changes noted on the basis of visual observations.

RESULTS

When the leaf segments were cultured onto MS medium supplemented with 2,4-D no morphogenic response was obtained irrespective of concentration (2-12µM) of hormone utilized however cultures leaves do exhibited enlargement in size. The leaves turned pale yellow and eventually dried out (Fig.2A-B). contrary to this, in an earlier study conducted by [11], development of callus from leaves of *R. serpentine* was achieved onto MS medium supplemented with 2,4-D. Medium supplemented with lower concentration of NAA (2-5µM) resulted in no response even after prolonged incubation but when concentration of NAA was increased upto 10-12µM leaves initially enlarged in size followed by callus development (Fig. 3A-B). Callus obtained was green and compact. Callus obtained onto this medium exhibited limited growth and did not proliferated further and eventually turned brown and dried out unless aseptically excised and subcultured.

Onto media supplemented with different/varying combination of auxin and cytokinin both direct as well as indirect regeneration from the cultured leaf segments was obtained.

Among different combination of auxin and cytokinin MS media supplemented with NAA and BAP was found to be most suitable media composition for morphogenic response.

When the leaf segments were cultured onto MS+4µM NAA+2µM BAP, initial crumbling and enlargement of leaves occurred which was eventually followed by callus development from the portion of leaves present in direct contact with culture medium. Callus development was achieved in about 52.1% cultures after about 40-45 days of incubation. After 8-10 days of callus initiation, enlargement and proliferation of callus was observed. Callus obtained was creamish green and granulated. Onto MS+8µM NAA+4µM BAP direct regeneration of shoots was achieved from cultured leaf segments. Initially the leaves enlarged in size (about 20 times the original size) followed by regeneration of shoots (Fig. 4A-B). Development of multiple shoots was achieved after 6-7 weeks of incubation in 68% cultures with a maximum of 15 shoots onto this medium (Table 1, Fig. 4C). Beside this callus formation was also achieved onto this media. Regenerated shoots were further elongated onto regeneration medium, MS+NAA+BAP (Fig. 5A) however if they were excised and transferred to basal medium individual shoots exhibited elongation (Fig. 5B) and could be easily excised and utilized for *in vitro* rooting.

Utilization of basal medium for elongation of *in vitro* regenerated shoots have been reported in tissue culture studies pertaining to other plants also [12]. From the observations made and results obtained in the present study it was found that young and growing leaves gave far better morphogenic response as compared to results obtained from *in vitro* culture of mature leaves.

As evident from literature available pertaining to tissue culture of several plant species, IAA and IBA are the two most commonly utilized PGR for *in vitro* rooting in *Rauwolfia* and other plants as well [13,14,15]. However, in the present study medium fortified with NAA was found to be extremely efficient for generation of *in vitro* roots (Table 2). In earlier studies [11] also it has been reported NAA to be the most effective PGR for *in vitro* rooting in *R. serpentine*. In the present study onto MS+ 10-20 μ M NAA, initially a single comparatively thick root protruded out from shoot portion dipped into media. Initially the root exhibited slow growth but after 3-4 weeks of root induction extensive branching and multiple root formation was achieved (Fig. 6A-B). The roots were prominent, thick in appearance. A maximum of 88.8% cultures exhibited *in vitro* rooting onto MS+20 μ M NAA, with an average of 12.2 roots per culture (Table 2). However with formation of well developed, highly branched roots onto NAA supplemented medium it was also observed that growth of shoots was comparatively slow. Besides that, development of *in vitro* roots was also achieved onto IBA supplemented medium in the present study. Effectiveness of IBA for *in vitro* root induction in *R. serpentine* have also been reported by other workers [11,16] as well. Irrespective of the type of hormone utilized, enhance root development was achieved onto ½ strength MS medium as compared to full strength medium. About 72.45 % regenerated plants survived during the process of transplantation (7A-B).

Table 1. Response of *in vitro* cultured leaf segment of *R. serpentine*

NAA (μ M)	BAP (μ M)	% Culture developing callus	Degree of callusing	% Culture developing shoots	Max. No. of shoots
2	-	44.6	++	-	-
4	-	57.4	++	-	-
8	-	74.6	+++	-	-
10	-	78.2	+++	-	-
2	2	48.4	++	-	-
4	2	52.1	++	-	-
10	2	48.4	+++	-	-
4	8	58.6	++	-	-
4	10	63.2	++	-	-
8	2	68.4	++	43.2	09 \pm 0.2
8	4	88.2	++	68.0	15 \pm 0.6
10	2	85.2	++	44.6	08 \pm 0.2
10	4	86.4	++	52.1	11 \pm 0.5

-No response; + poor; ++ good; +++ extensive

Table 2: *In vitro* rooting from regenerated shoots onto MS+NAA medium

NAA (μ M)	% Culture developing roots	Nature of roots	Avg. number of roots
2	-	-	-
5	-	-	-
10	68.6	Branched	5.6 \pm 0.6
15	82.4	Highly	9.8 \pm 0.4
20	88.6	branched	12.2 \pm 0.4



1A- Mother plant of *R. serpentina*, 2(A- B)- Leaf enlargement and drying out onto MS+2,4-D, 3(A-B)-Callus induction and proliferation onto MS+NAA, 4A- leaf enlargement onto MS+8µM NAA+4µM BAP, 4B- shoot bud induction from leaf segments, 4C- Multiple shooting along with callus formation; 5A- Elongation on MS+NAA+BAP, 5B- elongation of shoot onto basal medium, 6(A-B)- *In vitro* rooting onto MS+15µM NAA. 7(A-B)–transplanted plants.

CONCLUSION

The present study provides an efficient method of mass propagation of *R. serpentina*. Utilization of leaves as explants provides an advantage of minimum damage caused to mother plant as mother plant remains intact. Present protocol can be further analyzed to elicit molecular and biochemical changes occurring during direct and indirect organogenesis.

REFERENCES

- [1] Roy, S.K, Hossain, M.Z., Islam, M.S. 1994. Mass Propagation of *Rauwolfia serpentina* by *in vitro* Shoot Tip Culture. *Plant Tissue Cult.* 4:69-75.
- [2] Dey, A and De, J.N. 2010. *Rauwolfia serpentina* (L.) Benth.ex. Kurz.-AReview, *Asian Journal of Plant Sciences* 9: 285-298.
- [3] 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.
- [4] 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.

- [5] Nayar, M.P. and Shastry, A.R.K. 1990. Red Data Book of Indian Plants. Vol. 3, Botanical Survey of India, Calcutta.
- [6] 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 Sonbhadra, Uttar Pradesh, India. *Adv.Biol. Res.*4:65-80.
- [7] 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.
- [8] Chaudhary V, Singh S, Vaid S and Sharma N 2015. *In vitro* regeneration of *Rauwolfia serpentine* through another culture studies. *Biotechnology International.* 8: 93-100.
- [9] Deshmukh SR, Dhanashree S A, Patil BA 2012. Extraction and evaluation of indole alkaloids from *Rauwolfia serpentine* for their antimicrobial and antiproliferative activities. *Int J Pharm Pharm Sci.* 4. 329-334.
- [10] Murashige, T.S., Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-479.
- [11] Mallick SR, Jena RC and Samal KC 2012. Rapid *in vitro* multiplication of endangered medicinal plant Sarpagandha (*Rauwolfia serpentine*). *American J of Plant Sci.* 3:437-442.
- [12] Sharma N, Koshy EP and Dhiman M 2014. Indirect regeneration from leaf segments for mass Propagation of *Withania somnifera* :An endangered medicinal plant. *Int J Pharm Bio Sci.* 5: 764 – 772.
- [13] Bahuguna, R.N., R. Joshi., G. Singh., A. Shukla., R. Gupta., G. Bains 2011. Micropropagation and total alkaloid extraction of Indian snake root (*Rauwolfia*): an important antihypersensitive medicinal shrub. *Indian J. Agric Res.*, 81: 1124-1129.
- [14] 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.
- [15] Sharma N, Sachdeva P, Dhiman M and Koshy EP 2015. Comparative evaluation of *in vitro* regeneration potential of seeds of *W. somnifera* and *W. coagulans*. *Biotechnology International* 8 (1): 21-33.
- [16] 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.

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