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LEAF CALLUS CULTURE OF *RAUWOLFIA SERPENTINA*

IHSAN ILAHI AND MEHMOOD AKRAM*

Department of Botany,
University of Peshawar, Peshawar, Pakistan.

Abstract

Young leaves of *Rauwolfia serpentina* Baill., inoculated on MS medium supplemented with NAA @ 1 mg/l with 10 mg/l K and 10% CM under 24 h light induced callus while 2, 4-D induced callus under 16 h light. Callus induction and its growth was more on explants taken from *in vitro* raised seedlings on 2, 4-D @ 1 mg/l or NAA @ 1 mg/l + 10 mg/l K and 10% CM under 24 h light.On subculturing the callus exhibited good growth. Similarly leaf callus growth on AM medium containing NAA, BAP, K, AS and 2, 4-D with 1 g/l of CH showed excellent growth. The cultures on MS contained 0.0249-0.0281% ajmaline, and 0.041% on AM medium.

Introduction

Rauwolfia serpentina Baill., a tropical plant of woody nature belonging to the family Apocynaceae is one of the oldest medicinal plant which grows wild in India, Pakistan, Burma, Bangladesh, Srilanka and Malaya (Woodson *et al.*, 1957). The drug rauwolfia derived from the roots is used for the treatment of high blood pressure and as a tranquilizer. In Pakistan the plant has been grown to some extent in nurseries and medicinal plant farms.

Mitra *et al.*, (1965) obtained the leaf callus cultures of *R. serpentina* by activating the phloem and parenchyma around the vascular region of the leaf lamina. Callus cultures from leaf of *R. serpentina* were also reported by Mitra Chatrurvedi (1970) and the studies were confined to histogenesis and regeneration. There was need for establishing cultures of *R. serpentina* which could be maintained for certain growth periods. Studies were made to to find out whether the important constituents can be isolated from the leaf directly so as to reduce the long developmental phenomenon when the plant is destroyed after more than 5 years growth for securing the roots. In the present communication various media and growth hormones with two types of leaf explants of *R. serpentina* were used for callus initiation, proliferation of leaf callus and their chemical analysis.

Materials and Methods

Young leaves of *R. serpentina* from field plants and aseptically raised seedlings were used for inoculation. Plants were obtained from Medicinal Plants Farm of Pak-

*Tissue Culture Facility, Pakistan Council of Scientific & Industrial Research, Peshawar, Pakistan.

istan Forest Institute, Peshawar. Murashige & Skoog (1962) medium (MS) as modified by Gamborg & Wetter (1975) and Abou Mandour (1977) medium (AM) were used. The MS and AM media were supplemented with respectively, 2 and 3% sucrose, 0.8% agar, pH 5.8 and sterilized at 15 p.s.i. for 15 min. Napthalene acetic acid (NAA) 2, 4-dichlorophenoxy acetic acid (2, 4-D), kinetin (K), benzyl aminopurine (BAP) and adenin sulphate (AS) with and without casein hydrolysate (CH) or coconut milk (CM) were used at different concentrations.

Leaves were washed with tap water, surface sterilized with $HgCl_2$ for 3 min and rinsed thrice with autoclaved distilled water. About 20 mm segments were inoculated on solidified medium and inoculated at 25 ± 1°C under 16:8 light/dark cycle or 24 h continuous light regimes.

Calli to be analysed were chopped into pieces, freeze dried and stored at 20°C until extracted. Extraction of lyophillized, powdered calli was done twice with 40 ml 1.1 methanol: chloroform mixture for 2 h at 40°C. Extracts decanted and dried under vacuum and 70 µl equivalent to 1 gm tissue from each sample was applied on silicagel (Merck) thin layer chromatographic (TLC) plates in the form of 10 mm streaks at a distance of 5 mm. Acetone-methanol-acetic acid (70:25:5) was used as solvent for TLC and scanned at 290 and 306 nm for ajmaline and serpentine, respectively. The amounts were determined against separate standards for each of the constituents (Kraus & Stahl, 1980).

Results and Discussion

A. *Cultures Initiation:* Little amount of callus was produced after 56 days only in the cultures containing 2,4-D @ 0.1, 0.5 and 1 mg/l.

Copious callus formation was observed in treatments with 1 mg/l NAA + 10 mg/l K on explants taken from seedlings as compared to good callus on explants from field growing plants. Callus formation occurred on seedling leaves after 20 days of culture in comparison to 28 days for field plants (Fig. 1). No callus formed with NAA + K on leaves of field plants under 16 h illumination (Table 1).

Copious callus was formed with 1 mg/l 2, 4-D after 25 days on seedling leaf explants under 24 h illumination. On the other hand, when leaves from mature plants were kept under 16 h illumination and cultured with 2, 4-D callus was produced after 56 days but in less quantity (Table 1). It would appear that 24 h illumination did enhance certain metabolic processes to induce callus on leaf explants.

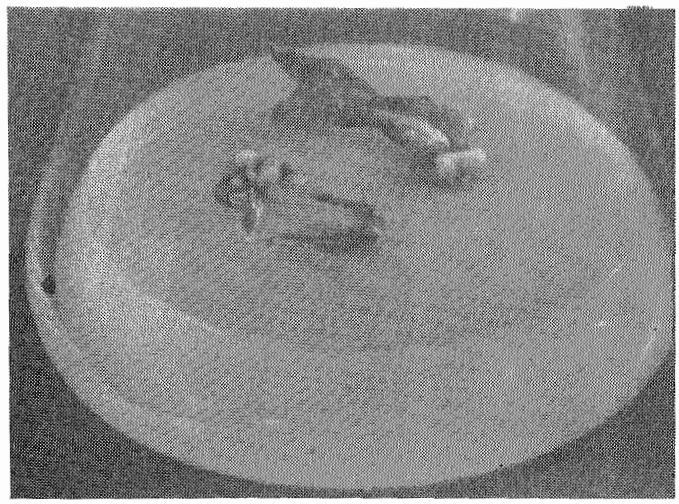

Fig. 1. Callus formation on leaf explants taken from field growing plants of *Rauwolfia serpentina*, cultured under continuous light, on MS medium, with 1 mg/l NAA, 10 mg/l K and 100 ml/l CM.

B. *Callus Propagation:* Callus initiated on leaves of field grown plants when cultured on MS medium containing 10 % CM with either 2, 4-D @ 1 mg/l or 1 mg/l NAA + 10 mg/l K and kept under 16 and 24 h light regimes exhibited very little growth after 42 days. The callus was not sufficient enough for further propagation.

Table 1. Callus formation on leaf explants of *Rauwolfia serpentina* on MS medium with different treatments of growth regulators and 10% coconut milk. The results are an average of 5 replicates for each treatement.

Explant origin	TREATMENT		RESULTS	
	Growth Regulators (mg/l)	Illumination	Callus Formation	after (Days)
Field Plants	–	16 h	–	–
	2, 4-D 0.1	–do–	+	56
	0.5	–do–	+	56
	1.0	–do–	+	56
	NAA 0.1	–do–	–	–
	0.5	–do–	–	–
	1.0	–do–	–	–
	NAA 1.0 + BAP 0.5	–do–	–	–
	NAA 1.0 + K 10.0	–do–	–	–
	–do–	24 h	++	28
Aseptic Seedlings	–do–	–do–	+++	20
	2, 4-D 1.0	–do–	+++	25

– no callus; + fair calls; ++ good callus; +++ Copious callus.

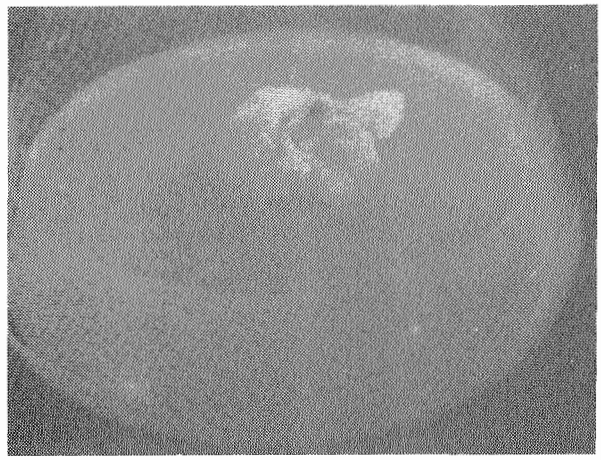

Fig. 2. Callus formation on leaf explants taken from aseptic seedlings of *Rauwolfia serpentina*, cultured under continuous light, on MS medium, with 1 mg/l 2, 4-D and 100 ml/l CM.

Callus obtained from seedling leaves cultured under 24 h illumination, either on MS with 10% CM and 1 mg/l of 2, 4-D or 1 mg/l of NAA + 10 mg/ K or on AM medium with 1 g/l CH and 2, 4-D, NAA, K, AS AND BAP @ 6, 1, 0.3, 4 and 0.1 mg/l respectively showed copious growth in all treatments (Table 2). Duration of copious growth was, 63, 70 and 91 days, respectively, for MS with 2, 4-D, MS with NAA + K and AM medium (Figs. 2-4).

In the present work, 2, 4-D used alone was found considerably effective for callus formation in leaf segments at various concentrations ranging from 0.1 to 1 mg/l than NAA. These results are in agreement with Staba (1969) who found 2, 4-D alone as an effective auxin mostly at a concentration of 1 mg/l or occassionaly at a higher concentration of 3 mg/l (Carew, 1966).

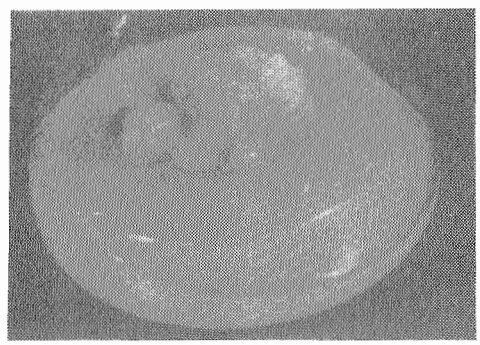

Fig. 3. Growth of leaf callus from aseptic seedlings of *Rauwolfia serpentina*, cultured under continuous light on MS medium with 1 mg/l NAA, 10 mg/l K and 100 ml/l CM.

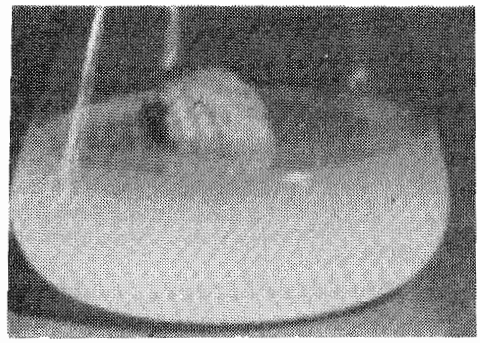

Fig. 4. *Rauwolfia serpentina* leaf callus growth by 4th week on AM medium supplemented with NAA, BAP, K, AS and 2, 4-D as 1, 0.1, 0.3, 4, 6 mg/l and 1000 mg/l CH.

Table 2. Growth in leaf callus of *Rauwolfia serpentina* on MS and AM media with different treatments of growth regulators, 10% coconut milk in all treatments except No. 7 (AM) which contain 1 g/l casein hydrolysate, and under varied light period. Analysis of copiously growing callus (Nos. 5-7) are given for serpentine and ajamline. Percentage was calculated on dry weight basis. The results are an average of 5 replicates for callus growth and 3 replicates for chemical analysis.

			TREATMENT				RESULTS	
No.	Explant origin	Medium	Growth Regulators (mg/l)	Illumination	Callus Growth	Growth Period (days)	Alkaloids (%) Serpentine	Ajmaline
1.	Field Plants	MS	2, 4-D 1.0	16 h	+	42		
2.		–do–	–do–	24 h	+	42		
3.		–do–	NAA 1.0 + K 10.0*	16 h	+	42		
4.		–do–	–do–	24 h	+	42		
5.	Aseptic Seedlings	–do–	–do–	–do–	+++	70	0.00686	0.0249
6.		–do–	2, 4-D, 1.0	–do–	+++	63	0.00489	0.0281
7.		AM	2, 4-D 6.0 + NAA 1.0 + BAP 0.1 + AS 4.0 + K 0.3	–do–	+++	91	0.00735	0.0410
8.	Control**						+	+

*Callus was initiated with 24 h illumination.

**Leaf sample taken from field growing parental stock of *R. serpentina.*

+Callus growth: negligible/alkaloids: traces only.

+++Copious callus growth.

Adenine sulphate, kinetin and benzylaminopurine had useful combined effect (total cytokinins = 4.4 mg/l) with a high concentration of 2, 4-D (6 mg/l) used in the present study in AM medium, for successful and continuous culture of leaf callus of *R. serpentina.* Our results agree with those of the combined effect of 2, 4-D with K (Dhoot & Henshaw, 1977), BAP (Javeed *et al.*, 1980), K + BAP + AS (Abou Mandour, 1977) and BA (Zainul-abedin *et al.*, 1977) on callus formation and callus growth in other plant species.

Coconut milk 10-15% (Mitra *et al.*, 1965), casein hydrolysate 500 mg/l (Perveen, 1978) and yeast extract 0.1 to 1% (Ohta & Yatazawa, 1979) has been used for formation and culture of callus in *R. serpentina.* In the present studies CM used as 10% (v/v) in MS medium was found suitable for callus formation in leaf segments, while CH at 1 g/l was found comparatively a little better than CM for continuous growth of leaf callus (Table 2).

C. *Chemical Analysis:* The cultures analysed showed 0.00686, 0.00489 and 0.00735% serpentine in calli growing on MS medium with NAA + K, 2, 4-D and AM medium, respectively. In the same cultures ajmaline was found at 0.0249, 0.0281 and 0.041% respectively (Table 2). The leaves of *R. serpentina* contain ajmaline and serpentine only in traces, while our results indicate that these two alkaloids were present in comparatively sufficient amounts in cultures. It would further suggest that therapeutic active alkaloid ajmaline which was previously not isolated from leaves of *R. serpentina* plants, due to its presence in traces only, can now be isolated from leaf cultures in sufficient amounts. The amount of ajmaline can be further enhanced in *Rauwolfia* cultures by hormonal manipulations and various changes in physical conditions required for culture growth than given by Stockigt *et al.*, (1981).

Acknowledgements

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References

Abou-Mandour, A.A. 1977. Ein standardnahrmedium fur die anzucht von kalluskulturen einiger arzneipflanzen. *Z. Pflanzen-physiol.*, 85: 273-277.

Carew, D.P. 1966. Growth of callus tissue of *Catharanthus roseus* in suspension culture. *J. Pharm. Sci.*, 55: 1153-1154.

Dhoot, G.K. and G.G. Henshaw. 1977. Organization and alkaliod production in tissue culture of *Hyoscyamus niger. Ann. Bot.*, 41: 943-949.

Gamborg, O.L. and L.R. Wetter. 1975. *Plant Tissue Culture Methods.* National Research Council of Canada. pp. 4-7.

Javeed, Q.N., E. Parveen, I. Haq and I. Ilahi. 1980. Propagation of *Juniperus polycarpos* C. Koch. through tissue culture I. Induction of callus. *Pak. J. Forestry*. 30: 72-77.

Kraus, Lj. and E. Stahl. 1980. Dunnschichtchromatographischen Nachweis Der *Rouwolfia* Alkolide In: *Arzneispezialitaten Und Drogenmaterial. Phormazeutische Biologie* II (C-2): 15-16.

Mitra, G.C. and H.C. Chatturvedi. 1970. Fruiting plants from *in vitro* grown leaf tissue of *Rauwolfia serpentina* Benth. *Curr. Sci.* 39: 128-129.

Mitra, G.C., C. Prabha and H.C. Chaturvedi. 1965. Histogenesis of callus tissue from different organs of *Rauwolfia serpentina* Benth. in tissue culture. *Indian J. Exptl. Biol.*, 3: 216-222.

Ohta, S. and M. Yatazawa. 1979. Growth and alkaloid production in callus tissues of *Rauwolfia serpentina. Agric. Biol. Chem.*, 43: 2297-2303.

Parveen, R. 1978. Some studies on callus formation in *Rauwolfia sepentina* Benth. M.Sc. Thesis. Deptt. Botany, University of Peshawar, Pakistan.

Staba, E.J. 1969. Plant tissue culture as a technique for the phytochemist. In: *Recent Advances in Phytochemistry* (eds.) M.K. Seikel, V.C. Runcekles, N.Y. Appleton. Century Crofts. Vol. 2, pp. 75-106.

Stockigt, J., A. Pfitzner and J. Firl. 1981. Indole alkaloids from cell suspension cultures of *Rauwolfia serpentina* Benth. *Plant Cell Rep.*, 1: 36-39.

Woodson, R.E., H.W. Youngken, E. Schlitter and J.A. Schneider. 1957. *Rauwolfia. Botany, Pharmacognosy, Chemistry and Pharmacology.* Little Brown and Company, Boston. p. 32.

Zain-ul-Abedin, M., A. Nafees and A. Mahmood. 1977. Tissue Culture studies on *Amaranthus viridis.* In: *Genetic Diversity in Plants.* (eds.) A. Muhammad, R. Aksel and R.C. von Borstel. Plenum Publ. Corp. N.Y. pp. 439-446.

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