ROOT CALLUS CULTURES OF *RAUWOLFIA SERPENTINA* BENTH.*

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Root explants obtained from mature plants and juvenile aseptic seedlings were subcultured for callus formation with 2,4-D, NAA and K. Explants taken from seedling roots gave the best response for callus formation on WRC medium containing 1 mg/1 2,4-D and 100 ml/1 CM, Callus propagation in subcultures was found good both on initiation medium and on AM medium with BAP, K, AS, NAA, 2,4-D and CH at 0.1, 0.3, 4.0, 1.0, 6.0 and 1000 mg/1. The alkaloids screened were serpentine, ajmaline, raubasine, raupine and reserpine. The major alkaloid present in cultures was ajamline. Its maximum percentage was 0.0573 % in the cultures grown under dark on AM medium, corresponding to an increase of 94.61 % over cultures kept in 16 hr light.

Key words: Rauwolfia serpentina, Root callus growth, Precursor, Ajmaline.

INTRODUCTION

Roots of *Rauwolfia serpentina* Benth. are used as raw material for the extraction of indole alkaloids, chiefly reserpine, ajmaline and ajmalicine. *R. serpentina* grows wild in India, Bangladesh, Sri Lanka, Burma, Malaya, Thailand and Java in moist deciduous forests ranging from sea level upto 1200 m.

The recent and rapid advances in the development of plant tissue culture techniques have allowed large scale cultivation of plant tissue and exploration of their biosynthetic capabilities. Rapidly growing suspension cultures of *Ruta graveolens* simultaneously produced volatile oils [1], furanocoumarins [2] and alkaloids [3].

Callus formation on root explants of various medicinal plant species have been reported, e.g. *Solanum laciniatum* [4], *Atropa belladonna* [5], *Panax ginseng* [6], *Glycyrrhiza echinata* [7], *Digitalis purpurea* [8], *Rosmarinus officinalis* [9] etc.

Mitra and Kaul [10] reported the formation of callus tissue from the radical of *R. serpentina* embryo cultured with 1 mg/1 2,4-D and 10 % CM.

The purpose of present studies is to initiate callus cultures from the root of *R. serpentina* and to see if there are any variations from plant root in chemical constituents. Moreover, root callus cultures would save 2 to 3 years [11] required for the accumulation of alkaloids in the roots of wild plants.

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MATERIAL AND METHODS

A. *Root explants.* Root explants of *Rauwolfia serpentina* were obtained from plants goriwng at PCSIR Laboratories, Peshawar. Seeds harvested at the Medicinal Plants Farm of Pakistan Forest Institute, Peshawar were used for growing aseptic seedlings.The roots of these juvenile seedlings were also used as explants.

For raising aseptic seedlings, seeds were washed thoroughly with tap water and floated in a beaker containing clean tap water. Heavy seeds settled down at the bottom of beaker. These seeds were selected and surface sterilized with 1 % solution of mercuric chloride containing 1 % Tween 80. Seed testae were afterwards manually cracked under aseptic conditions. Seeds were then inoculated on solidified WRC medium. The cultures were placed for seed germination at a temperature of 30 ± 1° in dark. After germination by the 3rd week, the cultures were left for one more week in dark and then incubated in 16 hr light. The temperature was lowered to 28°. After the 5th week of seed germination the cultures were provided with 10 % CM for better growth of seedlings.

Root explants taken from nursery grown mature plants were washed thoroughly with running tap water to clean the adhering soil. Afterwards the root segments were surface sterilized by treating them with 1 % solution of mercuric chloride and inoculated aseptically on solidified agar media.

B. *Culture media.* The following culture media were used :

(1) Murashige and Skoog's (MS) medium [12]. It was supplemented with 2 % sucrose. Vitamins added in MS

medium were according to B5 [13]. Calcium chloride was used at 2.9 ml/1 from a stock solution made by dissolving 15 g of $CaCl_2.2H_2O$ in 100 ml distilled water [13]. Ferric ethylenediamine tetraacetate (FeEDTA) was used at 7.84 mg/1.

(2) White's Root Culture (WRC) medium [14]. It was supplemented with 1.5 % sucrose. FeEDTA was used as per MS medium.

(3) Abou-Mandour's (AM) medium [15]. Sucrose was added at 3 %. The medium was added with CH at 1000 mg/1 and growth hormones BAP, K, AS, NAA and 2,4-D at 0.1, 0.3, 4.0, 1.0 and 6.0 mg/1 respectively.

The pH of various media was adjusted at 5.8 with 1N NaOH and 0.1N HCl. The media were solidified with 0.8 % agar. Sterilization of media was done by autoclaving at 1 kg per sq.cm pressure and 121° for 15 min.

C. *Chemical Analysis.* The callus to be analysed was chopped into pieces and squeezed into folds of filter papers to remove the water. Afterwards it was freeze dried for 10 to 12 hr and the dried callus was stored at -20°.

The callus before analysis was weighed, powdered and then extracted with 40 ml mixture of 1 : 1 methanol – chloroform in a water bath for 2 hr at 40°. It was then vacuum dried and applied to thin layer plates. The plates used were kieselgel Merck 60F 254 nm and acetone-methanol-acetic acid (70 : 25 : 5) was used as the solvent system. The material was applied as 70 µl per 10 mm length. The distance between two bands of the material applied was 5 mm.

Calculations were done on Scanner – Zeiss chromatogram calculating apparatus. The wave length used for ajmaline, serpentine and raubasine (ajmalicine) was 290, 306 and 280 nm respectively.

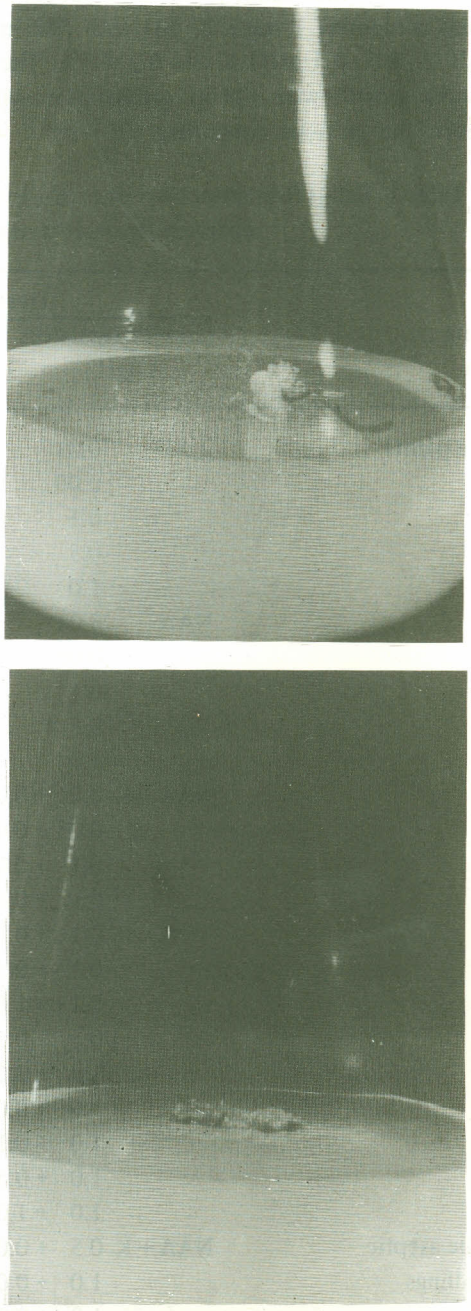
RESULTS

A. *Callus cultures.*

1. *Culture of root explants from mature plants.* Root segments were cultured on MS medium containing 1 mg/1 2, 4-D. No callus was formed on these roots upto 90 days. Thereafter, a low salt medium, WRC, was used, containing 500 mg/1 CH and 0.01, 0.05, 0.1, 0.5 and 1.0 mg/1 2, 4-D. Callus was seen initiating in 7 days in cultures containing 1 mg/1 2, 4-D. This callus was subcultured on WRC medium containing either 1 mg/1 2, 4-D or 0.05 to 1.0 mg/1 NAA in combination with K at 0.05 to 0.1 mg/1. The callus growth occurred in next 3 weeks with following treatments: 1 mg/1, 2, 4-D; (Fig. 1A) 0.05 mg/1 NAA and K; 0.5 mg/1 NAA and 0.05 mg/1 K; 1.0 mg/1 NAA and 0.01, 0.05 (Fig. 1B) and 0.1 mg/1 K.

Another set of root segments was inoculated with NAA at 0.01, 0.05, 0.1, 0.5 and 1.0 mg/1 in WRC medium with 500 mg/1 CH. Callus was observed after 21 days in cultures containing 1 mg/1 NAA.

Some root segments were given NAA in combination with K in WRC medium (Table 1). Callus formation was observed after 7 days in cultures containing 0.1, 1.0 mg/1 NAA with 0.01 mg/1 K and 1 mg/1 NAA with 0.05 mg/1



(A)

(B)

Fig. 1. Callus formation in 3 weeks on root explant taken from mature plant of *Rauwolfia serpentina* cultured on MS medium with 500 mg/1 CH and:

A) 1.0 mg/1 2,4-D

B) 1.0 mg/1 NAA and 0.05 mg/1 K.

K. In another 14 days the following treatments also showed callus initiation: 0.05, 0.1 mg/1 NAA and 0.05 mg/1 K; 0.1, 1.0 mg/1 NAA and 0.1 mg/1 K and 0.5 mg/1 NAA and K. Callus pieces were then subcultured for propagation purposes on WRC and MS media, each containing 500 mg/1 CH with either 1 mg/1 NAA and 0.01 mg/1 K or 0.5, 1.0 mg/1 NAA and 0.05 mg/1 K. No growth was seen in either of the above mentioned treatments after 10 weeks.

Thereafter, root callus pieces were subcultured on MS medium containing 1 mg/1 2, 4-D with 500 mg/1 CH or 1 mg/1 2, 4-D, 0.5 mg/1 K and 0.2 % YE. The callus did not show growth with CH in combination with 2, 4-D However, the cultures containing YE with 2, 4-D and K showed some growth for 7 weeks (Fig. 2). Afterwards, the growth was inhibited. Then 250 mg/1 SDC was added in cultures with 2, 4-D, K and YE. The callus started growth which continued upto 46 weeks (Table. 2).

2. *Culture of root explants from aseptic seedlings.* Root explants were cultured on WRC medium containing 0.5 and 1 mg/1 NAA with 0.05 mg/1 K and either with 500 mg/1 CH, 100 ml/1 CM or without CH/CM. Slight callus formation occurred in 63 days in cultures containing 500 mg/1 CH. This callus, however, did not proliferate further.

Another batch of cultures was provided with 1 mg/1 2, 4-D and 100 ml/1 CM in WRC medium. Root segments

Table 1. Callus formation on root explants of *Rauwolfia serpentina* on White's Root Culture (WRC) medium with different treatments of growth regulators, 500 mg/1 CH and 100 ml/1 CM

Explant origin	Treatment: Growth regulators (mg/1)		Treatment: Nutrients	Callus: Formation (quality)	Callus: Initiation (days)
Mature plants	2,4-D	0.01	CH	–	–
		0.05	CH	–	–
		0.1	CH	–	–
		0.5	CH	–	–
		1.0	CH	+	7
	NAA	0.01	CH	–	–
		0.05	CH	–	–
		0.1	CH	–	–
		0.5	CH	–	–
		1.0	CH	+	21
	NAA + K	0.01 + 0.01	–	–	–
		0.05 + 0.05	–	+	21
		0.1 + 0.01	–	+	7
		0.1 + 0.05	–	+	21
		0.1 + 0.1	–	+	21
		0.1 + 0.5	–	–	–
		0.1 + 1.0	–	–	–
		0.5 + 0.5	–	+	21
		1.0 + 0.01	–	+	7
		1.0 + 0.05	–	+	7
		1.0 + 0.1	–	+	21
		1.0 + 0.5	–	–	–
		1.0 + 1.0	–	–	–
Juvenile aseptic seedlings	NAA + K	0.5 + 0.05	CM	–	–
		1.0 + 0.05	CM	–	–
		1.0 + 0.05	CH	+	63
	2,4-D	1.0	CM	++	14

Legend: – no callus, + fair callus, ++ good callus.

in these cultures initiated callus in 14 days (Fig. 3). Callus proliferated copiously and exhibited active growth for 14 weeks. The callus appeared as compact and white. Thereafter, root callus turned slightly brown with slowing down of growth. The root callus was further subcultured on a high salt medium: AM, containing 2, 4-D, K, BAP, AS and NAA at 6.0, 0.3, 0.1, 4.0 and 2.0 mg/1 with 1000 mg/1 CH. Subcultures on AM medium showed active growth for 40 weeks (Table 2) and were of yellow-white colour (Fig. 4A). Another batch of cultures on AM medium was kept under dark. Here the callus growth was observed as slow than light grown cultures. The callus looked as a hyaline mass (Fig. 4B) and grew for 50 weeks.

B. *Chemical analysis*

The root of *R. serpentina* plants, under cultivation, was screened for the presence of serpentine, ajmaline, raubasine, raupine and reserpine. The alkaloids serpentine, ajmaline and raubasine were present at 0.1252, 0.0642 and 0.0536 % respectively. Raupine and reserpine were present in traces only.

Fig. 2. Root callus showing growth on MS Medium supplemented with 0.2 % YE, 1.0 mg /1 2,4-D and 0.5 mg/1 K.

Table 2. Growth in root callus of *Rauwolfia serpentina* on White's Root Culture (WRC), MS and Abou-Mandour (AM) media with different treatments of growth regulators, 100 mg/1 CM, 500/1000 mg/1 CH, 2000 mg/1 YE and 250 mg/1 SDC.

Callus origin	Treatment			Callus		
	Medium	Growth regulators (mg/1)		Additives	Growth	Growth period (weeks)
Mature plants	WRC	2,4-D	1.0	CH	+	3
		NAA+K	0.05 + 0.05	–	+	3
			0.5 + 0.05	CH	–	–
			0.5 + 0.05	–	++	3
			1.0 + 0.01	CH	–	–
			1.0 + 0.01	–	++	3
			1.0 + 0.05	CH	–	–
			1.0 + 0.05	–	++	3
			1.0 + 0.05	CM	–	–
			1.0 + 0.1	–	+	3
	MS	NAA+K	0.5 + 0.05	CH	–	–
			1.0 + 0.01	CH	–	–
			1.0 + 0.05	CH	–	–
		2,4-D	1.0	CH	–	–
		2,4-D+K	1.0 + 0.5	YE	+	7
			1.0 + 0.5	YE+SDC	+	46
	AM	2,4-D+K+NAA + BAP + AS	6.0 + 0.3+1.0+ 0.1 + 4.0	CH*	–	–
Juvenile aseptic seedlings	AM	– do –	– do –	CH*	+++	40
	WRC	2,4-D	1.0	CM	++	14

Legend: – no growth, + fair growth, ++ good growth, +++ copious growth, * CH was used at 1000 mg/l.

In callus cultures from the root of parental stock, the percentage of serpentine was found at 0.0043 % in cultures growing on MS with 0.2 % YE, 1 mg/1 2, 4-Dand 0.5 mg/1 K. The percentage of ajmaline was 0.0156 for these cultures. The samples could not be analysed for raubasine. Raupine and reserpine were absent in these cultures.

The percentage of serpentine was at 0.0088, 0.0069, 0.0042 and 0.0084 respectively for cultures growing on AM medium with 1,5 and 10 mg/1 tryptophan. The percentage of ajmaline was 0.0507, 0.0546, 0.0498 and 0.0292 respectively. The percentage of raubasine for these cultures was 0.0071, 0.0093, 0.0065 and 0.0175 respectively. Raupine was found in traces in cultures growing without tryptophan. Reserpine was absent in these cultures.

In dark grown AM cultures, the percentage of serpentine, ajmaline and raubasine was 0.0121, 0.0573 and 0.0114 respectively. Raupine and reserpine were absent in these cultures.

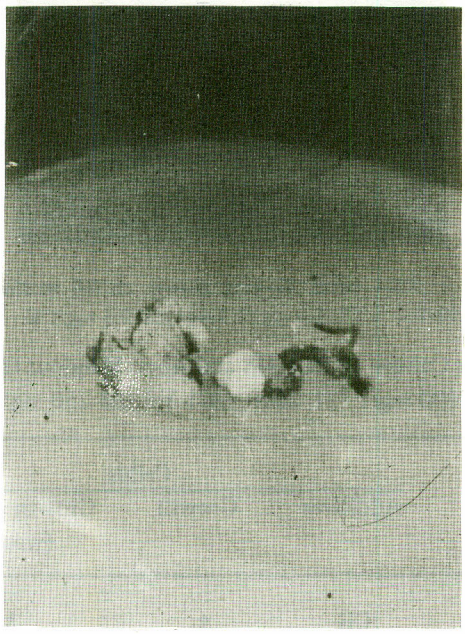
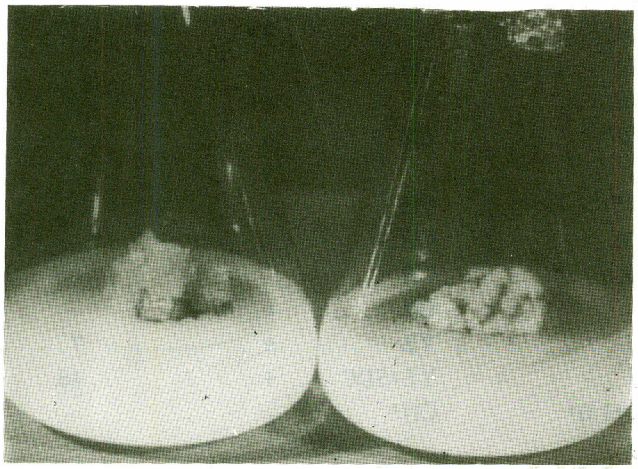


Fig. 3. Root explant from juvenile aseptic seedling formed callus in 14 days on White's Root Culture, medium containing 100 ml/1 CM and 1.0 mg/1 2,4-D.



(A) (B)

Fig. 4. Root callus showing growth on Abou-Mandour medium:
(A) Callus growth in 16 h light as yellow-white
(B) Callus growth in dark as a hyaline mass.

DISCUSSION

It is evident from the present studies that a low salt medium, e.g. White's Root Culture (WRC) was required for the induction of callus on root segments of *R. serpentina.* However, high salt media MS/AM were required for callus proliferation for longer duration (Table 2). Mitra and Kaul [10] also preferred White's medium for induction of callus on radicals of *R. serpentina.* The auxin, 2, 4-D used at 1 mg/1 with MS and WRC media produced callus in 7 days on low salt (WRC) medium against 90 days on high salt (MS) medium.

It was observed that 2, 4-D was a better auxin than NAA for callus induction in the present studies as NAA at 1 mg/1 produced callus on WRC medium in 21 days. However, when NAA was given in combination with K, it was effective at 1 mg/1 with 0.01 to 0.05 mg/1 K to initiate callus in 7 days. The synergistic effect of NAA with K was almost similar both for root explants taken from mature plants or from juvenile aseptic seedlings.

The propagation of callus obtained on root segments from mature plants was not feasible on WRC medium. However, it gave good growth for 46 weeks on MS medium (Table 2). Nonetheless, the callus obtained on root segments of aseptic seedlings grew copiously both on low salt (WRC) medium i.e. 14 week active growth and on high salt medium (AM) for 40 to 50 weeks. On AM medium in dark grown cultures, the active growth period of callus was found for longer duration (50 weeks) than light grown cultures (40 weeks). However, the growth rate, i.e. quantity of callus mass produced per subculture period was a bit low in dark grown cultures.

The cultures contained mainly ajmaline. Maximum amount of ajmaline was found at 0.0573 % in root callus grown on AM medium in dark. In light grown cultures on AM medium, the percentage of ajmaline was only 0.0292. Therefore, in dark grown cultures the increase in ajmaline was 94.61 % over cultures growing in 16 hr light. Moreover, the amount of ajmaline with 5 mg/1 tryptophan, a precursor of indole alkaloids, was 0.0546 which is an increase of 86.98 % over cultures grown without tryptophan. It is

Table 3. Percentage of indole alkaloids serpentine, ajmaline and raubasine, on dry weight basis, in root callus cultures of *Rauwolfia serpentina.*

Culture Medium	Growth regulators (mg/1)		Organic N Nutrients (mg/1)	Precursor L-tryptophan (mg/1)	Light/dark cultures	Serpentine	Ajmaline	Raubasine
MS	2, 4-D + K	1.0 + 0.5	YE 2000	–	Light 16 h	0.0043	0.0156	n.e
AM	2, 4-D + K + RAP + AS + NAA	6.0 + 0.3 + 0.1 + 4.0 + 1.0	CH 1000	–	–do–	0.0084	0.0292	0.0175
”	–do–	–do–	–do–	1	–do–	0.0088	0.0507	0.0071
”	–do–	–do–	–do–	5	–do–	0.0069	0.0546	0.0093
”	–do–	–do–	–do–	10	–do–	0.0042	0.0498	0.0065
”	–do–	–do–	–do–	–	Dark	0.0121	0.0573	0.0114
Plant root						0.1252	0.0642	0.0536

Legend: – nil, n.e. not evaluated.

interesting to note that the quantitative increase in ajmaline was found better in cultures growing simply in dark and without precursor than in cultures growing with tryptophan but in routine experimental conditions of illumination. Higher concentration of tryptophan at 10 mg/1 was inhibitory to ajmaline in comparison with 5 mg/1 (Table 3). The plant roots contained a little higher percentage of ajmaline, i.e. 0.0642 % as against maximum percentage of 0.0573 in callus cultures. However, we believe that physical and chemical manipulations in cultures could enhance the quantity of ajmaline from that found in cultivated plants of *R. serpentina.*

Ohta and Yatazawa [16] observed that ajmaline contents in root callus of *R. serpentina* were strikingly reduced when 2, 4-D concentration was increased than 1 mg/1. On the other hand, we observed an increase of 46.57 % with increase in 2, 4-D concentration from 1 mg/1 (MS medium) to 6 mg/1 (AM medium) (Table 3).

The quantity of serpentine and raubasine are much low in cultures than found in cultivated plants. However, cultural manipulation still provides a chance to increase the percentage of serpentine and raubasine, as could be seen in dark grown cultures for serpentine and in cultures grown in ligh/dark (without precursor) for raubasine (Table 3).

In the light of the present studies, it may be concluded that alkaloidal contents, particularly the percentage of ajmaline, in the root callus cultures of *R. serpentina* could be increased by cultural manipulations.

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