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An Economical Approach towards Optimization of Organic Media for Callus and Cell Suspension Culture of *Rauvolfia serpentina*

Research Article

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Abstract

Protocol for callus induction using the leaf explant of *Rauvolfia serpentina* was standardized using formulated organic vermicompost extract and coelomic fluid (extracted from the earthworms *Eudrilus eugeniae*) of various combinations used, 30% vermicompost + 4% coelomic fluid was found to be the best for callus induction. Copious, shiny white callus was observed after two weeks; further creamy white detachable callus resulted after sixth week of culture. This on comparison with Murashige and Skoog medium supplemented with different combinations of BAP (1 mg L-1) + IBA (0.125 mg L-1) and BAP (1.0 mg L-1) + 2, 4-D (0.125 mg L-1) was made respectively. Of the different ratios tried, 3:1 ratio of vermicompost extract: coelomic fluid was found to be best for initiating cell suspension cultures. Phytochemical analysis reported in 34.83±0.14 mg/g of total phenols and 0.063±0.002 mg/g of total flavonoids from the extracted calli and cell suspension samples. Reserpine is detected as the major alkaloid in the callus as well in cell suspension culture (15.151 retention times in HPLC analysis). These phytochemicals produced by in vitro cultures can be significantly used for pharmaceutical purpose. Present study manifested significant callus development on organic vermicompost extract (30%) media and its economic value.

Keywords: *Rauvolfia serpentine*; Economic growth media; Vermicompost extract as media; Coelomic fluid as supplement; Reserpine

Abbreviations

Abb1: 2,4–D- 2,4-Dichlorophenoxyacetic acid; Abb2: IAA - Indoleacetic acid; Abb3: IBA - Indolebutyric acid; Abb4: BAP - 6-benzylaminopurine; Abb5: NAA - Naphthaleneacetic acid; Abb6: KIN - Kinetin; Abb7: MS - Murashige and Skoog; Abb8: GAE s - Gallic acid equivalents; Abb9: QE - Quercetin Equivalent; Abb10: EDTA - Ethylenediaminetetraacetic acid; Abb11: IUCN - International Union For Conservation Of Nature.

Introduction

Rauvolfia serpentina (Linn.) Benth, is well recognized as sarpagandha, belongs to Apocynaceae family. It is small, woody, perennial medicinal shrub and is most commonly used in Ayurvedic, Unani, Siddha and Western Medicines [1]. This snake-weed genus includes 50 species. It is wide ranging in tropical part of Indian Peninsula, Himalaya, Indonesia, Sri Lanka and Burma. *R. serpentina* is aboriginal to India, Bangladesh and few regions of Asia. A root of *R. serpentina* comprises of fifty indole alkaloids which includes pharmaceutically important alkaloids viz., ajmaline, deserpidine, reserpine, rescinnamine, and yohimbine. The research found that the natural stock of medicinal plant *R. serpentina* is indiscriminately exploited in India by pharmaceutical sectors. *R. serpentina* was listed as endangered by International Union for Conservation of Nature

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(IUCN). The alkaloid reserpine was used as sedative or tranquilizing agent and also to treat hypertension [2-14].

Micropropagation of *R. serpentina* and callus formation has been reported by many plant tissue culturists. Optimization of macro salts concentrations in the synthetic tissue culture media has also been reported. Callus culture from leaf of R. serpentina [13,26,34,40], reserpine in cell culture [42], somatic embryogenesis and plant regeneration have also been reported using MS media. Liquid medium was standardized for tissue culture of *R. serpentina* [13,30]. Direct root induction from leaf explants and effect of growth regulators were as well studied. Alkaloid formation in hairy roots and suspension cell cultures and techniques like Thin Layer Chromatography and High Performance Liquid Chromatography were established for separation and quantification of alkaloids (15,23,10,28]. Vermicompost extract is known to possess humic and fulvic substances that promote plant growth and resistance to various diseases [9,20]. Coelomic fluid of earthworm has been found to possess strong hemolytic, agglutinating and bacteriostatic activities [38]. The present study is focused towards establishment and standardization of callus and cell suspension culture system from leaf explant of *R. serpentina* using formulated organic media (vermicompost and cooelmic fluid). Further phytochemical analysis was done to assess the presence of compounds of pharmaceutical importance. Main objective of the study is organic media optimization, phytochemical comparison of the *in vitro* callus and *in vivo* plants.

Materials and Methods

Collection of Explants

Tender and disease free leaves of *Rauvolfia serpentina* (Linn.) Benth were collected and authenticated by Dr. Rajanna, taxonomist, University of Agricultural Sciences (UAS), Gandhi Krishi Vignan Kendra (GKVK), Bangaluru, INDIA.

Preparation of Media and culture techniques

Murashige and Skoog (1962), medium (Sigma Chemicals) was used as the control medium. MS medium with 5.6-5.8 pH, 3% sucrose was solidified with 9 mg/L plant grade agar and supplemented with different combinations of growth hormones respectively.

Vermicompost was produced using earthworms- *Eudrilus eugeniae* on organic waste mix of plant litter, vegetable waste and cow dung slurry. Vermicompost (30%) thus obtained was suspended in sterile distilled water and agitated for 8 h and the aqueous extract (filtrate) consisting of humic and fulvic acids obtained is used after 24 h. The pH maintained is 5.8 and supplemented with agar (9 g/L). Parallelly, coelomic fluid was collected from earthworms- *Eudrilus eugeniae*, using chemical method (5% chilled ethanol and 2.5 mg/ml of EDTA). Thick straw colored liquid coelomic fluid thus obtained was used in media as a supplement.

MS medium (control) and organic vermicompost extract medium were sterilized under standard autoclave conditions. After sterilization, MS media bottles were supplemented with different combinations of filter sterilized BAP (1 mg/L) + IBA (0.125 mg/L) and BAP (1.0 mg/L) + 2, 4-D (0.125 mg/L) respectively. Parallelly organic vermicompost media bottles were supplemented with filter sterilized 4% coelomic fluid for callus induction.

Leaf explants of R. *serpentina* were surface sterilized using Tween 20 [5% (v/v) for ten minutes], 70% ethanol (30 s to 1 min), mercuric chloride [0.1% (w/v) for 2 to 3 min] and finally washed thoroughly with sterile distilled water for several times. The sterile leaf explants of *R. serpentina* were soaked in the autoclaved vermicompost extract and coelomic fluid (in 3:1 ratio) for 3-5 min to avoid the release of phenols in the culture bottles. Sterile leaf explants of 1 to 2 cm of *R. serpentina* were then inoculated into the organic medium and control MS media bottles for callus induction.

Cultures were initiated in baby jar bottles containing 25 ml of medium. The MS medium cultures were regularly subcultured on fresh MS medium at 4 weeks intervals in baby jar bottles. Whereas the vermicompost media bottles containing callus were subcultured at 8th week. Observations were recorded every 3 days following inoculation and subculturing. Parallelly, calli obtained from the organic vermicompost media survived without subsequent subculturing like in MS media calli. All experiments were repeated twice with at least 25 cultures per treatment. Callus obtained from the MS media and organic vermicompost media were then used in establishing cell suspension cultures respectively.

Cell Suspension Culture

Suspension cell culture was initiated by inoculating 1 g of six weeks old *R. serpentina* leaf callus obtained from the organic vermicompost extract media into 125 mL Erlenmeyer flask containing 25 mL liquid vermicompost extract and coelomic fluid in 3:1 ratio under aseptic conditions. The flasks were subjected to continuous shaking on the rotary shaker at 100 rpm for 24 h at 25 ± 2°C [21]. The cell culture obtained was subjected to filtration. Filtered cells were weighed and further used for phytochemical analysis.

Phytochemical Analysis

Around 100 mg of freeze dried callus and cell suspension cultures were extracted using 5 mL methanol for 20 min in Soxhlet apparatus. The crude extract was treated with 0.01 M HCl and then filtered. The pH of the filtrate was adjusted to 6.0 with 0.01 M NaOH. The extracted and powdered sample was screened for phytochemical contents like phenols, flavonoids using spectrophotometric analysis and alkaloids using TLC and HPLC methods.

Determination of Total phenols and flavonoids

The concentration of total phenols and flavonoids in the *in vitro* (callus and cell suspension cultures) and naturally grown (control) plant sample extracts was determined using Spectrophotometric method [31,37]. The samples were analyzed in triplicates and the mean values were recorded.

Thin Layer Chromatography and High Performance Liquid Chromatography

TLC analysis was performed on preparative silica gel-60 plates using chloroform: methanol (97:3) solvent systems for the separation of alcoholic extracts of callus and the cell suspension cultures of *R. serpentina* obtained from the vermicompost extract medium. The peaks obtained for callus, cell suspension extract and suspension

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medium (filtrate) were recorded. For qualitative purpose, the method was evaluated by taking into account the Retention factor (Rf). Acetonitrile: Phosphate Buffer (35:65) was used as the mobile phase. Wavelength was detected at 268 nm and 20 µL of the sample was injected with the flow rate of 1 mL min^{-1}. This protocol was performed at ambient temperature and retention time of 20 min was obtained. Isocratic method was implemented for obtaining chromatograms of alkaloids from the callus and cell suspension cell cultures of *R. serpentina*.

Cost analysis

Cost analysis [22] was done to assess the economic implications observed between usage of MS medium and formulated organic vermicompost extract media. Expenses incurred in preparing the 1L MS with hormones and formulated organic vermicompost media were calculated and the differences were noted and economics were concluded.

Statistical Analysis

Statistically, ANOVA and Student t-test were applied to note the significant differences in the growth of callus in MS medium and formulated organic vermicompost extract media. Total phenolics and flavonoid content in callus cultures and naturally grown plant samples of *R. serpentina* were comparatively analyzed. Significance of the study was proven by "P" values having less than 0.05.

Results

Callus Induction in R. serpentina among the various combinations experimented; survival rate calculated is as follows. 100% callus induction was obtained without contamination in organic media with 30% vermicompost and 4% coelomic fluid, whereas only 40% callus induction was observed in the control media after 6 weeks of culture and the same is recorded in Table 1.

Callus induction was initiated within one week of inoculation of leaf explant of *R. serpentina* on vermicompost extract medium without chemical supplementation. Two weeks after inoculation, white copious shiny callus developed on the vermicompost extract medium. Callus formation was slower in the initial days of culture. Slowly callus covered the entire media within four weeks (Figure 1B). Low cost vermicompost extract medium was economical and 100 % survival was recorded. Sub culturing of callus using vermicompost extract medium containing 2 mg/L BAP + 1 mg/L 2,4-D was used to sustain the continuous growth of the callus and also to reduce contamination. Excellent detachable callus was observed on the pre-existing callus after six weeks of culture. Creamy white callus covered the major portion of explant.

Table 1: Effect of media and its chemical supplements on callus induction from leaf explants of R. serpentina.

Media	Combination of phytohormones concentration (mg/L)			Percentage response of callus induction	Number of times sub-cultured	Observation
	BAP	IBA	2,4- D			
MS	1.0	0.125	-	30	2	Callus
MS	1.0	-	0.125	40	2	Callus
Vermi compost extract only	-	-	-	100	4	Callus
Vermi compost+ coelomic fluid (3:1)	-	-	-	98	4	Callus

Phytochemical screening

Spectrophotometric analysis for total phenols and flavonoids was found to be 34.83±0.14 mg/gram and 0.063±0.002 mg/gram respectively in callus when compared to 71.03±0.53 mg/gram of phenols and 0.26±0.002 mg/gram of flavonoids in naturally grown extracted plant samples (Table 2). The Fluorescent green and the blue bands observed on preparative silica gel plates when exposed to ultraviolet light reported the presence of alkaloid derivatives present in the sample extract. TLC showed Retardation factor (Rf) value of 0.45, this was in very close proximity with standard Reserpine. From TLC analysis, reserpine was found to be the major alkaloid in the

Table 2: Comparative analysis of total phenolic and total flavonoid content present in *in vivo* plants and *in vitro* suspension cultures of *Rauvolfia serpentina* and their t- statistical values.

Sample	*In Vitro*±SE	*In vivo* ±SE	t statistical value
Total Phenol Content in mg/g	34.83±0.14	71.03±0.53	0.0000**
Total Flavonoid content in mg/g	0.063±0.002	0.26±0.002	0.000002**

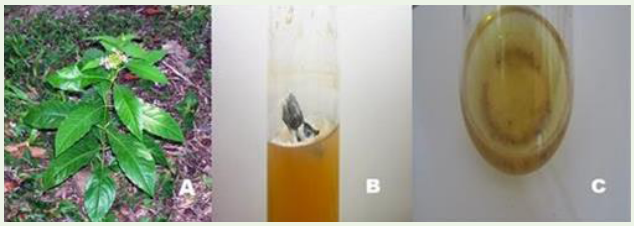

Figure 1: A: *R.serpentina*; B: Growth response of R. serpentina cultured on vermicompost extract media along with coelomic fluid experimented for callus development; C: Suspension cell culture of R. serpentina.

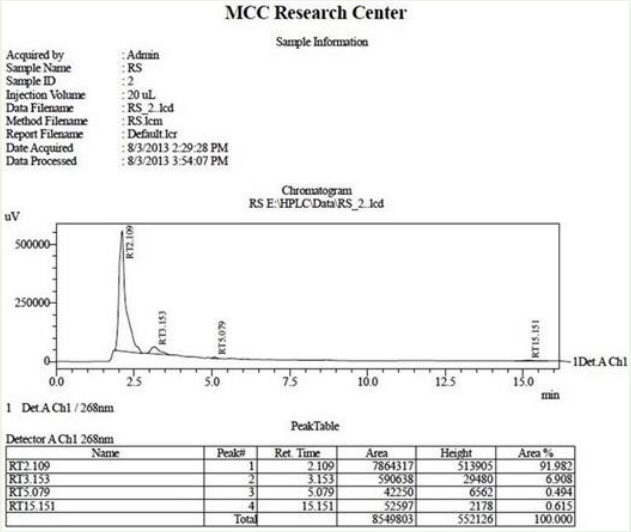

Figure 2: Chromatogram of alkaloids depicting Reserpine as a major alkaloid from suspension cell extract of R. serpentina developed using organic vermicompost extract medium supplemented with coelomic fluid.

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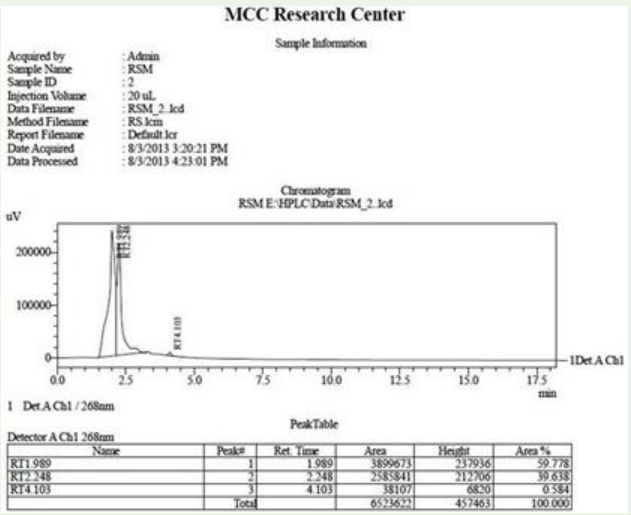

Figure 3: Chromatogram of alkaloids detected from the suspension media (vermicompost and coelomic fluid in 3:1 ratio) developed for suspension cell culture of *R. serpentina* and the peak is comparable to that of the alkaloid peak of the cell extract.

sample extracts. The peaks obtained against the respective retention time indicated the presence of alkaloids. Reserpine peak was observed at 15.151 retention time from the sample extract of cell suspension and callus cultures (Figure 2). Callus and cell suspension culture almost deciphered similar results; a peak at 2.109 min indicated the presence of an alkaloid ajmaline (Figure 2).

Cost Analysis

The expenditure incurred for preparation of one litre MS medium along with growth regulators is Rs.66.58/– whereas for the organic formulated vermicompost extract medium is Rs.10.28/-. This indicates that organic media is economically feasible for medicinally important plants [21].

Discussion

Callus development from leaf explants on MS medium with various combinations of 2,4- D + BAP, 2,4-D + KIN and NAA + BAP were reported earlier [35]. Development of callus on MS medium containing 1or 2 mg/L BAP + 1 mg/L 2,4-D or 2 mg/L BAP + 1 mg/L IAA. Successful achievement of organogenic callus was reported on using 2 mg/L BAP + 1 mg/L 2,4 -D in MS Medium. Similarly, 93.65% of callus was resulted on MS medium along with 2 mg/L 2,4 -D + 1 mg/L BAP, whereas MS basal medium alone was unsuccessful for multiple shoots formation. MS with 1.5 - 2 mg/L BAP elicited in multiple shoot formation and the percentage of shoot induction ranges between 22.87-56%. On the other hand, rooting attained 100% on MS medium with 0.2 mg L-1 NAA + 0.2 mg/L IBA [29]. Desirable callus was observed on MS medium augmented with 0.125 mg/L IBA and 1.0 mg/L BAP [4]. When MS medium was supplemented with 2.0 mg/L BAP + 1.0 mg/L IAA, meristemoid-like structures were noticed. Callus was well formed on MS medium along with 1 mg/L NAA + 0.5 mg/L KIN [18].

In the present research study, formulated organic vermicompost medium have proven to be highly conducive for callus induction from the leaf explants of *R. serpentina* and is one of the significances of this protocol. Well regimented, economical protocol was systematized for plant tissue culture of jeopardized, red listed, medicinal plant *R. serpentina*. The sterile, juvenile leaf explants were inoculated onto MS medium consisting of various combinations of plant growth promoters. Earlier the frequency of callus induction on the leaf explants of *R. serpentina* was found to be the highest of about 77.77% in MS medium containing 1.0 mg/L BAP + 0.5 mg/L IAA. Persistence of shoot formation was high of 75% in MS medium with 2.5 mg/L BAP + 0.4 mg/L IAA, and root formation was 100% in MS medium with 2.5 mg/L BAP + 0.5 mg/L IAA + 0.5 mg/L NAA. The survival rate of plantlets after hardening was 67% [13].

In vitro tissue cultures are effective in producing pharmaceutically important alkaloids also contains a spectrum of such metabolites which are similar to those present naturally in the *in vivo* plant [5]. Naturally existing (*in vivo*) primitive alkaloid contents of roots of *R. serpentina* was reportedly higher when compared to the *in vitro* callus [35]. Earlier 1.86±0.11 of phenols and 1.72±0.11 of flavonoids were well documented [16]. In the present research study, phytochemical analysis reported that total phenols were found to be 34.83 ± 0.14 mg/g and flavonoids to be 0.63 ± 0.002 mg/g in *in vitro* calli. This study as well concluded that phenols and flavonoids were higher in the *in vivo* that is naturally grown plant extracts when compared with *in vitro* callus and cell suspension extracts of *R. serpentina*. Reserpine has been reported as the major alkaloid by TLC, especially in the roots [8,25,32,33]. The presence of indole alkaloid derivatives indicated the presence of ajmaline, ajmalicine, yohimbine and reserpine. In addition, other two indole alkaloids viz. renoxydine and reserpine were as well reported in the callus masses. Reserpine being extracted from the plant samples collected from distinct places was detected in HPLC at 16.596 min RT [25].

Degree of callus formed in dark was significant from the leaf explants of *R. serpentina* cell suspension cultures provided good response and HPLC analyses for the qualitative estimation of alkaloids have shown significant outcome.

It is absolute necessity to preserve our natural medicinal plant resources, their rational and sustainable use, and their conservation, firmly in the arena of public health policy and concern. As the agricultural lands are shrinking and the natural medicinal plant habitats are disturbed, it is necessary to increase the application of plant tissue culture technology and its development on the conservation and sustainable use of medicinal plants. It is also a requisite to turn down the cost of production of micro propagules. In most cases innovation requires encouragement and financial support. Cost analysis was also considered for the study undertaken, that concluded vermicompost being economical [22].

Plant Tissue culture of *R. serpentina* proved exigent for most plant tissue culturists. Present study manifested significant callus development on organic vermicompost extract (30%) only media without any other chemical supplements.

Callus development of *R. serpentine* on vermicompost media was most probably due to hormone-like activity of humic acids present

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in the vermicompost. The study has shown that by standardizing the technique, it is possible to establish the plants and their alkaloids using tissue culture technology in an economical way.

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Conflict of interest

"The authors declare no financial or commercial conflict of interest."

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