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An Economical Approach Towards Optimization Of Organic Media For Callus And Cell Suspension Culture Of *Rauvolfia serpentina*

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Abstract

Protocol for callus induction using the leaf explant of R. serpentinawas 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. Copius, shiny white callus was observed after two weeks, further creamy white detachable callusresulted after sixth week of culture. This on comparison with control Murashige and Skoog medium supplemented with different combinations of BAP (1mg/L)+IBA (0.125mg/L) and BAP (1.0 mg/L)+2, 4-D (0.125mg/L) was made respectively. Different ratios of vermicompost extract: coelomic fluid tested, cell suspension was found to be best at 3:1 ratio. In callus, 34.83±0.14 mg/gramof phenolics and 0.26±0.002mg/gram of flavonoids were reported.Reserpine reported major alkaloid in the callus as well in cell suspension culture (15.151retention time in HPLC analysis).These phytochemicals produced by in vitro cultures can be significantly used for pharmaceutical purpose.

Keywords:*Rauvolfia serpentina*, Economical growth media, Vermicompost extract as media, Coelomic fluid as supplement, Reserpine.

Abbreviations: **Abb1:** 2,4 –D- 2,4- Dichlorophenoxy acetic acid; **Abb2:** IAA – Indole acetic acid; **Abb3:** IBA – Indole butyric acid; **Abb4:** BAP – 6-benzyl amino purine; **Abb5:** NAA – Naphthalene acetic acid; **Abb6:** KIN –Kinetin; **Abb7:** MS – Murashige and Skoog; **Abb8:** GAE s – Gallic acid equivalents; **Abb9:** QE – Quercetin Equivalent; **Abb10:** EDTA – Ethylenediaminetetra acetic 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 (Ajayi *et al.*, 2011). .This snake-weed genus includes 50 species. It is widely distributed in the tropical part of the Indian Peninsula, Himalaya, Indonesia, Sri Lanka and Burma. *R. serpentina* is indigenous to India, Bangladesh and other regions of Asia and is grown wild in many places around the country(Ghani, 1998). A root of *R. serpentina* comprises of fifty indole alkaloids which includes pharmaceutically important alkaloids viz., ajmaline, reserpine, rescinnamine, deserpidine and yohimbine. In India, *Rauvolfia* is threatened because of haphazad usage and exploitation of natural resources for commercialization by pharmaceutical industry and its limited cultivation (Gupta, 1989). *R. serpentina* was listed as endangered by International Union for

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Conservation of Nature (IUCN). The alkaloid Reserpine was used as sedative or tranquilizing agent and also to treat hypertension (Ford and Moyer, 1953; Vida, 1953;Bhatia, 1942; Chakraverti *et al*., 1951; Anonymous, 1950).

Micropropagation of *R. serpentina* (Mitra and Kaul, 1964; Butenka, 1964; Vollosovich and Butenka, 1970; Kukreja *et al.,* 1989 and Roy *et al.,* 1994) and callus formation(Perveen R, Ilahi, 1978) has been reportedby many plant tissue culturists.Optimization of macrosalts concentrations in the synthetic tissue culture media(Vollosovich *et al.,* 1979) has been reported.Callus culture (Ilahi and Akram, 1987) from leaf of *R. serpentina*, reserpine in cell culture(Yamamoto O, Yamada, 1986),somatic embryogenesis and plant regeneration(Ilahi *et al.,* 1995) have also been reported using MS media. Liquid medium was standardized for tissue culture of*R. serpentina*(Goel *et al.,* 2007). Direct root induction from leaf explants and effect of growth regulators(Pandey R, Mishra, 2010) were as well studied. Alkaloid formation in hairy roots and suspension cell cultures (Falkenhagen *et al*., 1993) and techniques like TLC and HPLC were established for separation and quantification of alkaloids (Habib and Court, 1974; Klushnichenko *et al*., 1994). Vermicompost extract is known to possess humic substances that promote plant growth and resistance to various diseases(Kale *etal*., 1987; Edwards and Burrows, 1988). Coelomic fluid of earthworm have been found to possess strong hemolytic, agglutinating and bacteriostatic activities(Valembois *et al*., 1982).The present study aimed to establish and standardize callus and cell suspension culture system from leaf explant of *R. serpentina* using organic media (vermicompost and cooelmic fluid). Further phytochemical analysis was done to assess the presence of compounds of pharmaceutical importance. The study was an attempt to use the low cost and economically feasible organic media formulated using vermicompost extract and coelomic fluid for *in vitro* plant tissue culture stuides.

2. Materials and Methods

2.1 Collection of Explants

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

2.2 Preparation of Media and culture technqiues

2.2.1 Callus culture

Murashige and Skoog (1962), medium (Sigma Chemicals)was used as the control medium. MS medium with 5.6-5.8 pH, 30% sucrose was solidified with 9g/L plant grade agarand supplemented with different combinations BAP (1mg/L)+IBA (0.125mg/L) and BAP (1.0 mg/L)+2, 4-D (0.125mg/L) respectively.

Vermicompost was produced using earthworms- *Eudrilus eugeniae* on organic waste mix of plant litter, vegetable waste and cowdung slurry. Vermicompost (30%) thus obtained was suspended in sterile distilled water and agitated for 8 hours and the aqueous extract (filtrate) obtained is used after 24 hours. The pH maintained is 5.8 and supplemented with agar (9g/L).Parallely, coelomic fluid was collected from earthworms- *Eudrilus eugeniae*, using chemical method(5 per cent chilled ethanol and 2.5 mg/ml of EDTA). Thick straw coloured liquid coelomic fluid thus obtained was used for media supplementation.

Control MS medium and Organic vermicompost extract medium were sterilized under standard autoclave conditions. After sterilization, MS media bottles were supplemented with different combinations of BAP (1mg/L)+IBA (0.125mg/L) and BAP (1.0 mg/L)+2, 4-D (0.125mg/L)

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respectively and 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 per cent (v/v)for ten minutes], 70 per cent ethanol(for 30 seconds to one minute), mercuric chloride [0.1 per cent (w/v) for2 to 3 minutes]and finally rinsed with sterile distilled water for several times.The sterile leaf explants of *R.serpentina* were soaked in theautoclaved vermicompost extract and coelomic fluid (in 3:1 ratio) for 3-5 minutes to avoid the release of phenols in the culture bottles. Sterile leaf explants of *R. serpentina*was then inoculated into the organic medium and control MS media bottles for callus induction.

2.2.2 Cell Suspension Culture

Suspension Cell culture studies were initiated by inoculating one gram of *R. serpentina* leaf callus obtained by previous protocol, into 125mL Erlenmeyer flask containing 25mL 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 hours at 25 ± 2 ^{0} C (Kashyap *et al.,* 2015).The cell culture obtained was subjected to filtration. Filtered cells were weighed and further used for phytochemical analysis.

2.3 Phytochemical Analysis

Callus and Cell suspension cultures were freeze-dried.100mg of dried sample wassubjected to Soxhlet apparatus for extraction using 5mL methanol for 20 minutes. The crude extract wastreated 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 samplewas screened for phytochemical contents like phenols, flavonoids and alkaloids. Phenols and flavonoids were determined using Spectrophotometric analysis and alkaloids usingTLC and HPLC methods.

2.3.1 Determination of Total phenols

The concentration of total phenols in the *in vitro* (callus and cell suspension cultures) and *in vivo* (control) plant sample extracts was determined using Spectrophotometric method(Singleton*et al.,* 1999). The samples were analyzed in triplicates and the mean values wererecorded.

2.3.2 Determination of Total flavonoids

The concentration of total flavonoids in the *in vitro* (callus and cell suspension cultures) and *in vivo* (control) plant sample extracts was determined using Spectrophotometric method (Quettier-Deleu *et al.,* 2000). The samples were analyzed in triplicates and the mean values were recorded.

2.3.3 Thin Layer 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.*

2.3.4High Perrformance Lqiuid Chromatography

From TLC analysis, Resepine was found to be the major alkaloid in the sample extracts. Qualitative analysis of Reserpine was performed using reverse phase HPLC under suitable experimental conditions and the peaks for callus, cell suspension extract and suspension medium (filtrate) wasrecorded. For qualitative purpose the method was evaluated by taking into account the Retention factor. 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 1mL/minute. This protocol was performed at ambient temperature and retention time of 20 minutes was obtained.

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Isocratic method was implemented for obtaining chromatograms of alkaloids from the callus and cell suspension cell cultures of *R. serpentina.*

2.4Cost analysis

Cost analysis (Kashyap and Kale, 2016)was done to assess the economic implications observed between usage of MS medium and organic formulated vermicompost extract media.

2.5Statistical Analysis

Statistically, ANOVA and Studentt-test were used to note the difference in the growth rate of callus induction MS and Vermicompost extract media. Total phenols and Total flavonoids present in *in vivo* plants and *in vitro* cultures of *R. serpentina* were comparatively analyzed using Student "t" test. Significance of the study was proven by "P" values having less than 0.05.

3. Results and Discussion

3.1 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 slowerin 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 per cent survival was recorded. Sub culturing of callus using vermicompost extract medium containing 2mg/L BAP+ 1mg/L 2,4-D was usedto sustain the continuous growth of the callus and also to reduce contamination. Excellent detachable callus was observed on the pre-existing callus aftersix weeks of culture.Creamy white callus covered the major portion of explant.

3.2 Cell suspension culture

Suspension Cell culture studies were initiated by inoculating one gram of *R. serpentina* leaf callus resulted in the formation of independent cells which covered the entire suspension medium. The cell cultures obtained was subjected to filtration. Filtered cells were weighed and further used for phytochemical analysis.

3.3 Phytochemical screening

3.3.1 Phenols: Spectrophotometric analysis for total phenol content was found to be 34.83±0.14mg/gram in *in vitro* callus when compared to 71.03±0.53mg/gram in *in vivo* plantsas shown in table 2.

3.3.2Flavonoids: Total flavonoid content was found to be 0.063±0.002mg/gram *in vitro* callus and 0.26±0.002mg/gram/ *in vivo* plants as shown in table 2.

3.3.3 Alkaloids: 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 extracted samples. TLC showed Retardation factor (Rf) value of 0.45, this was in very close proximity with standard Reserpine.

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

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callus cultures (Figure 2). Callus and cell suspension culture almost deciphered similar results; a peak at 2.109 minutes indicated the presence of an alkaloid ajmaline (Figure 2).

3.4 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.

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(Sehrawat*et al.,* 2001). Development of callus on MS medium containing 1or2mg/L BAP+1mg/L 2,4-D or 2mg/L BAP+1mg/L IAA. Successful achievement of organogenic callus was reported on using 2mg/L BAP +1mg/L 2,4-D in MS Medium. Response of callus was excellent with 93.65 per cent on MS medium supplemented with 2mg/L 2,4-D+1mg/L BAP. Multiple shoots failed to develop from the stem and leaf callus on basal MS medium. The MS medium with 1.5-2mg/L BAP resulted in multiple shoot formation and the shoot induction percentage ranged between 22.87-56 per cent. 100 per cent rooting was achieved when the MS medium was supplemented with 0.2mg/L NAA and 0.2 mg/L IBA(Panwar and Attitalla, 2011). Similar to present investigation, callus development was better on MS medium with 0.125mg/L IBA+1.0 mg/L BAP (Bhatt *et al.*, 2008)**.** Meristemoid-like structures appeared when MS medium was supplemented with 2.0 mg/L BAP+1.0 mg/L IAA. Similarly, callus development was better on MS medium with combination of 1 mg/L NAA+0.5 mg/L KIN (Ilahi and, Akram, 1993).

Efficient standardized protocol was developed for *in vitro* regeneration of endangered, red listed medicinal plant *R. serpentina*. The sterile, juvenile leaf explants were inoculated onto MS medium consisting of various combinations of plant growth promoters. The frequency of callus induction on the leaf explant of *R. serpentina* was the highest of about 77.77 per cent in MS medium containing 1.0 mg/L BAP+0.5 mg/L IAA. The frequency of shoot regeneration was highest with 75 per cent in MS medium containing 2.5 mg/L BAP+ 0.4 mg/L IAA. The frequency of root regeneration was 100 per cent in MS medium containing 2.5 mg/L BAP+0.5 mg/L IAA+0.5 mg/L NAA. The survival rate of plantlets after hardening was 67 per cent(Singh *et al.,* 2009).*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(Bohm, 1980). The crude alkaloid fraction was reported to be higher in roots of *R. serpentina* as compared to the *in vitro* callus (Sehrawat *et al.,* 2002). The phenol content of 1.86±0.11 and flavonoid content of 1.72±0.11 were as well reported (Harisaranraj*et al.,* 2009). In the present study, total phenolic (34.83±0.14 mg/gram) and flavonoid (0.26±0.002mg/gram) content were higher in the *in vivo* plants as compared to *in vitro* callus or cell suspension extracts. TLC analysis have shown the Reserpine as the major alkaloid in the roots (Court and Timmins, 1975; Roja *et al.,* 1984: 1987; Kumar *et al.,* 2010)as well as in the callus tissues. The presence of indole alkaloid derivatives indicated the presence of ajmaline, ajmalicine, yohimbine and reserpine. In addition, other two indole alkaloids viz. renoxydine and reserpigine were as well reported in the callus masses. Similarly, reserpine peak at 16.596 minutes retention time from extract of *R. serpentina* plant collected from different locations was reported(Kumar *et al.,* 2010).

Maintaining culture room temperature and placing the culture bottles in complete darkness supported the callus development in short span of time. Cell suspension cultures provided good response and HPLC analyses for the qualitative estimation of alkaloids have shown significant outcome.

In most cases innovation requires encouragement and financial support. To increase the application of plant tissue culture technology in the preservation of endangered medicinal plants, it is necessary to reduce the cost of micropropagule production. Cost analysis was carried out during this study which confirms that the vermicompost is economical (Rs. 10.227/- per litre) when compared with conventional MS medium (Rs. 66.576/- per litre) used in plant tissue culture studies(Kashyap *et*

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al., 2016). It can reach farmers at affordable prices and to develop in agricultural fields for mass production.

Tissue culture of *R. serpentina* is most challenging aspect. However Callus development reported to be better with vermicompost extract only without any other chemical supplements. And this study also confirms that 30% vermicompost have given the best result.

Callus development of *R. serpentina* on vermicompost media was most probably due to hormone-like activity of humic acids present 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. Further modifications in culture medium may provide the expected levels of the compounds for *in vitro* production of this economically important endangered plant.

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Conflict of interest

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

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Table 1: Effect of media and its chemical supplements on callus induction from leaf explants of *Rauvolfia 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
Vermicompost extract only	-	-	-	100	4	Callus
Vermicompost +coelomic fluid (3:1)	-	-	-	98	4	Callus

Table 2 Comparative analysis of total phenolics and total flavonoids present in *in vivo* plants and *in vitro suspension cultures* of *Rauvolfia serpentina* and their t- statistical values

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

Note: * Significance at 5per cent level

** Significance at 1per cent level

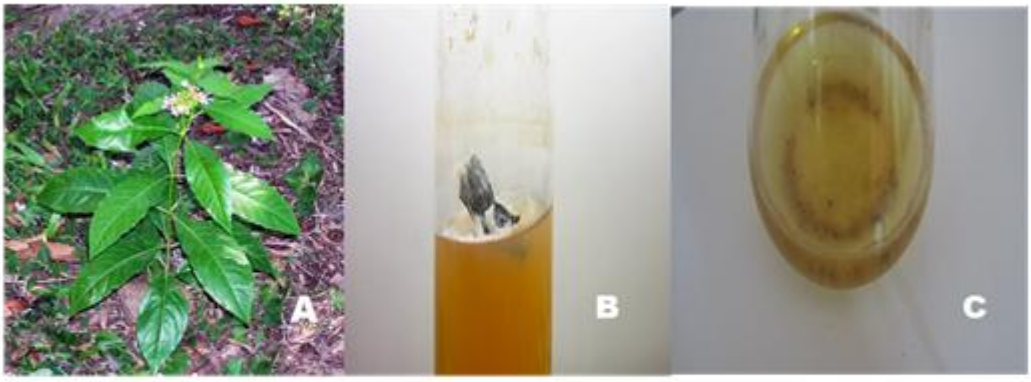

Fig 1: A: *R. serpentina*; **B:** Growth response of *R. serpentina* cultured on vermicompost extract media along with coelomic fluid experimented for callus development; **C:** Suspension cell culture of *R. serpentina*.

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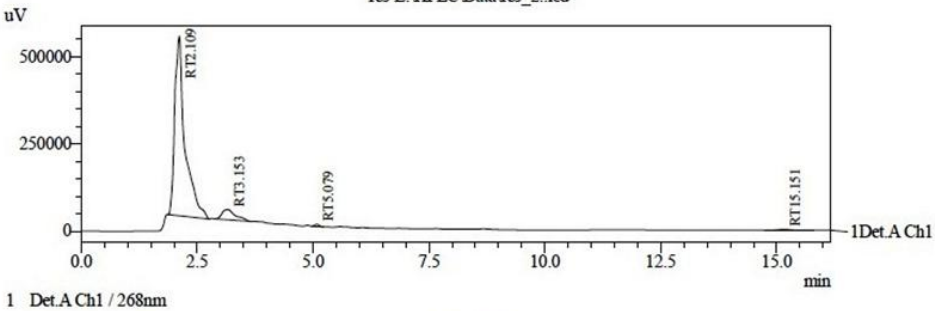
MCC Research Center

Sample Information

Acquired by	: Admin
Sample Name	: RS
Sample ID	: 2
Injection Volume	: 20 uL
Data Filename	: RS_2..lcd
Method Filename	: RS.lcm
Report Filename	: Default.lcr
Date Acquired	: 8/3/2013 2:29:28 PM
Data Processed	: 8/3/2013 3:54:07 PM

Chromatogram
RS E:\HPLC\Data\RS_2..lcd



1 Det.A Ch1 / 268nm

PeakTable

Detector A Ch1 268nm

Name	Peak#	Ret. Time	Area	Height	Area %
RT2.109	1	2.109	7864317	513905	91.982
RT3.153	2	3.153	590638	29480	6.908
RT5.079	3	5.079	42250	6562	0.494
RT15.151	4	15.151	52597	2178	0.615
	Total		8549803	552126	100.000

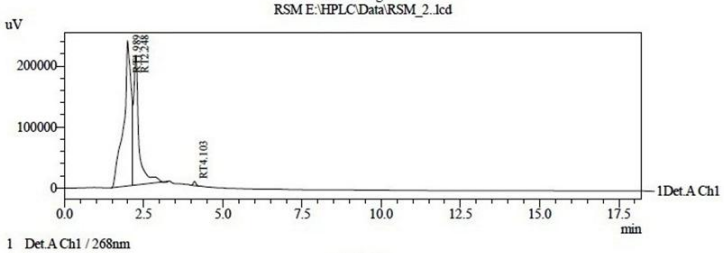
Fig 2 Chromatogram of alkaloids depicting Reserpine as a major alkaloid from suspension cell extract of *R. serpentina* developed using organic vermicompost extract medium supplemented with coelomic fluid.

MCC Research Center

Sample Information

Acquired by	: Admin
Sample Name	: RSM
Sample ID	: 2
Injection Volume	: 20 uL
Data Filename	: RSM_2..lcd
Method Filename	: RS.lcm
Report Filename	: Default.lcr
Date Acquired	: 8/3/2013 3:20:21 PM
Data Processed	: 8/3/2013 4:23:01 PM

Chromatogram
RSM E:\HPLC\Data\RSM_2..lcd



1 Det.A Ch1 / 268nm

PeakTable

Detector A Ch1 268nm

Name	Peak#	Ret. Time	Area	Height	Area %
RT1.989	1	1.989	3899673	237936	59.778
RT2.248	2	2.248	2585841	212706	39.638
RT4.103	3	4.103	38107	6820	0.584
	Total		6523622	457463	100.000

Fig 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.