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RESEARCH ARTICLE

IN VITRO TISSUE CULTURE OF *CATHARANTHUS ROSEUS* USING VERMICOMPOST EXTRACT AND COELOMIC FLUID - AN INNOVATIVE AND NOVEL APPROACH

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ABSTRACT

The study was initiated to develop callus using vermicompost extracts and coelomic fluid of earthworm *Eudrilus eugeniae* on *in situ* development of medicinal plant *Catharanthus roseus*. Vermicompost extract without any hormone supplements supported 100% callus induction. Vermicompost extract with 0.5mg/L BAP, 1mg/L NAA and 1mg/L IBA have supported 70% callus induction. Vermicompost extract with 1mg/L 2,4-D and 1mg/L KIN have shown 60% callus induction. Suspension culture was successfully developed using vermicompost extract and coelomic fluid in the 3:1 ratio. Phytochemical analysis has shown Total Phenol Content to be less in *in vitro* callus when compared to *in vivo* (86.88±0.12 mg/g) plants and flavonoids were higher (1.74±0.15 mg/gram) in callus. TLC and HPLC analysis have shown the presence of alkaloids in the plant calli and suspension cultures of *C. roseus.* Alkaloids recovered from the suspension media have indicated that certain important alkaloids can be obtained from the media itself without sacrificing the cells and the plants for pharmaceutical purposes. Vermicompost extract alone has given the best result. By standardizing the technique, it is possible to develop the plants through micropropagation in an economical way.

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INTRODUCTION

Catharanthus roseus (Madagascar periwinkle) or *Vinca rosea* L. belongs to family Apocynaceae, is an herbaceous shrub. It is a species of *Catharanthus* that is native and endemic to Madagascar. It is also called *Vinca rosea, Ammocallis rosea* and *Lochnera rosea*. Other English names include Cape Periwinkle, Rose Periwinkle, Rosy Periwinkle and "Old - maid". It is widely cultivated and also grows wild in subtropical and tropical areas of the world. Tissue culturing of medicinal plants is widely used to produce active compounds for herbal and pharmaceutical industries (Sidhu, 2010). *In vitro* propagation of plants holds a tremendous potential for producing high quality plant based medicines (Murch *et al.,* 2000). Various *in vitro* techniques as micropropagation from existing and adventitious meristem or organ, tissue and cell cultures provide a large amount of *C. roseus* plant material for the isolation of alkaloids exhibiting medicinal properties (Pietrosiuk *et al.,* 2007).

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A simple, efficient and reproducible regeneration system for *in vitro* propagation of *C. roseus* via nodal explants cultured on MS medium supplemented with different concentrations of BAP, NAA and IBA was also reported (Faheem *et al.,* 2011). In the present investigation, vermicompost extract along with the coelomic fluid (vermiwash) extracted from the body cavity of earthworm *Eudrilus eugeniae* were tested for *in vitro* culture and suspension cell culture of *C. roseus.*

MATERIALS AND METHODS

Collection of Medicinal Plant Sample (Explants)

Certified disease-free medicinal plants were obtained from Botanical garden of University of Agricultural Sciences (UAS), GKVK, Bangalore. Plants were planted in potted soil in the green house at the institution.

Preparation of the Media

The MS medium was purchased from Sigma Chemicals and final volume (1000ml) was made up with distilled water. About 20 ml of the medium was poured into sterile culture bottles. Fresh vermicompost (30%) was suspended in sterile distilled water and was placed on a stirrer for continuous

agitation for 8hrs. After 24hrs, aqueous extract comprising humic and fulvic acids was used to prepare the medium. This was supplemented with 9g/L of agar. Five per cent of ethanol and 2.5 mg/ml of EDTA under cold conditions were considered for coelomic fluid extraction. Known weights (31.5 grams) of earthworms were placed in this solution for 10-15 minutes. Thick straw coloured liquid of coelomic fluid was used for media preparation. Coelomic fluid collected from earthworm *Eudrilus eugeniae* and vermicompost extract were taken in 3:1 ratio for suspension media preparation. Coelomic fluid was used as spray in the *in vitro* propagation studies and was also used in 0.1-10 mg/L concentration in the vermicompost media as a growth regulator.

Protocol

pH of the media were maintained at 5.8-6.0. The concentrated stocks of the growth regulators were prepared and stored under refrigeration. The culture bottles with MS medium and vermicompost extract medium were autoclaved at 121°C for 20 min. at 15 lbs pressure and transferred to the media storage room where they were kept under aseptic conditions for further experimental study. The explants were washed under running tap water for 30 minutes and were treated with 5 per cent (v/v) detergent solution (Teepol) for 10 minutes followed by a rinse under running tap water. Aseptically explants were treated with 70 per cent alcohol for one minute followed by 0.1 per cent (w/v) mercuric chloride for 5 minutes. Further explants were washed thoroughly with sterile distilled water three to four times and leaves were removed using sterile blade. Sterile explants of C. *roseus* were inoculated into MS medium and vermicompost extract media containing 0.5mg/L of BAP, 1mg/L of NAA and 0.1mg/L of IBA. The leaf explants of *C. roseus* soaked in the autoclaved vermicompost extract and coelomic fluid (3:1 ratio) for 10 minutes were inoculated into only vermicompost extract media. The culture bottles were properly capped and sealed. The labelled bottles were transferred to the incubation room and incubated at 25± 2°C on racks covered with black paper.

Callus Induction

For callus development leaf segments and nodal segments were used as sources of explant. Callus growth was monitored by measuring fresh and dry weight of the developed callus.

Suspension cultures

Cell suspension cultures were initiated by inoculation of one gram of fresh leaf callus of selected plant into a 125 ml Erlenmeyer flask containing 25 ml of liquid vermicompost and coelomic fluid in 3:1 ratio. The flasks were placed on the rotary shaker at 100 rpm at 25 ± 2 ^{0}C in dark. No growth regulators were supplemented into the suspension media. The cells were separated from the medium by filtration for sub culturing and extraction of alkaloids.

Phytochemical Evaluation

Callus / Suspension cell culture weighing one gram was oven dried and extracted with 95% ethanol for 72 hours at 60 °C in Soxhlet extractor. The solvent was recovered by distillation in vacuum, and the residue was stored in the desiccator for subsequent experiments. Qualitative and quantitative estimations of phenols, flavonoids and active alkaloids were confirmed by Spectrophotometric analysis, TLC and HPLC analysis.

Thin Layer Chromatography

The extracted alkaloids from the *in vitro* micropropagated plantlets were used for TLC evaluation (Kurth, 1964; Stahl, 1969; Stock, 1974). In this evaluation, concentrated ethanolic extract was spotted on the precoated Silica gel 60 GF_{254} E. Merck plates of uniform thickness of 0.2 millimeter. The chromatogram was developed up to 80 millimeter under chamber saturation conditions with ethyl acetate: hexane, 30per cent (v/v) in a twin trough chamber and the plate was exposed to iodine vapours for visualization.

High Performance Thin Layer Chromatography

The secondary metabolite content was assessed using HPLC following the modified procedure described by (Deepak, 2005). The solvent was dried on rotary evaporator and then volume was made up to 2 ml. The sample was injected into HPLC column after filtering through 0.22 micron filter. 20 µl of the sample was injected into the HPLC system containing isocratic solvent system: phosphate buffer solution of pH 6.5 and acetonitrile (55:45). Retention time of 3.591 minutes was obtained. The flow rate was 0.5 ml/minute and the wavelength of 220 nm was maintained. The peak identity was confirmed by comparing the retention time of the experimental peaks with their standards.

RESULTS AND DISCUSSION

Callus development was observed within 15 days and roots emerged from 20 days old calli of *C. roseus. S*imultaneously shoot was also formed and finally the leaves were seen (Plate 1). Shoot and well developed root formation was observed from the callus on the vermicompost extract medium without any supplementation. Growth rate of callus formed on various media are tabulated in Table 1.

After four weeks of incubation, callus developed from the leaf segments on vermicompost extract media was sub cultured on the MS media and vermicompost extract media supplemented with 0.5mg/L of BAP, 1 mg/L of NAA and 0.1 mg/L of IBA which further supported shoot growth. New roots regenerated in the same media containing 1mg/L of 2, 4-D and 1 mg/L of KIN (Table 1). Suspension cultures were established using vermicompost extract and coelomic fluid in 3:1 ratio and leaf callus was used.

Phytochemical analysis

Standard alkaloids detected in the *in vitro* micropropagated plantlets grown on vermicompost extract showed the pattern of alkaloids with their respective retardation factors- serpentine (0.1), vincristine (0.25), vinblastine (0.35), cataranthine (0.75), vindoline (0.85), Ajmalicine (0.95).

Table 1. Effect of Media and its chemical supplements on callus induction from cultured leaf of *C. roseus*

SL No.	Media	Combination of phytohormones concentration (mg/L)					Percentage response of callus induction	Number of times sub cultured	Observation
1.	MS	0.5	1.0	0.1	-	-	45	2	Callus
2.	MS	-	-	-	1.0	1.0	60	2	Callus
3.	Vermicompost extract	0.5	1.0	0.1			70	4	Callus
4.	Vermicompost extract	-	-	-	1.0	1.0	60	4	Callus
5.	Vermicompost only	-	-	-	-	-	68	3	Callus
6.	Vermicompost extract only	-	-	-	-	-	100	4	Callus+shoots +roots
7.	Vermicompost+ coelomic fluid (3:1)	-	-	-	-	-	98	4	Callus
8.	Vermicompost extract (explants given coelomic fluid spray)	-	-	-	-	-	90	4	Callus

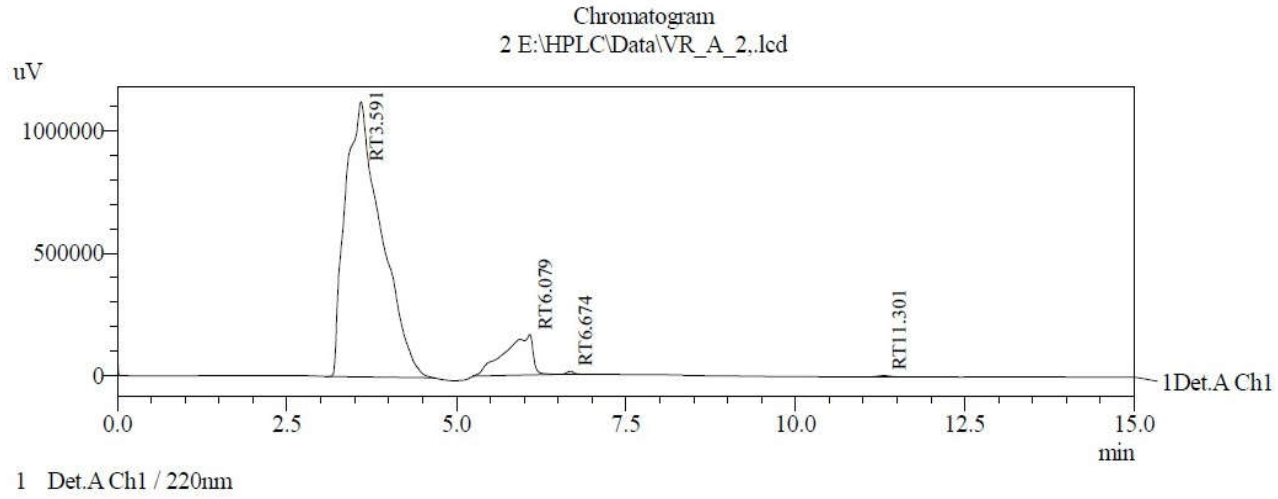

Fig 1. The Chromatogram of alkaloids detected from the *in vitro* grown suspension cells on Vermicompost extract media supplemented with coelomic fluid which is comparable with the standard alkaloids of *C. roseus*

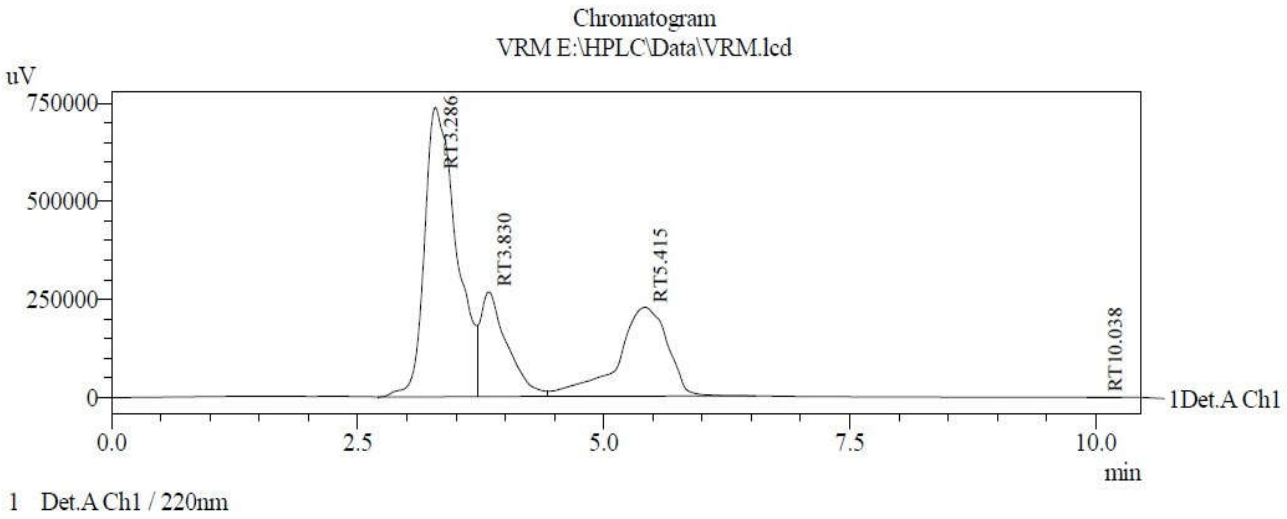

Fig 2. The chromatogram of alkaloids detected from the suspension media developed for suspension cell culture of *C. roseus* and the peak is comparable to that of the alkaloid peak of the cell extract

HPLC analysis (Fig 1) showed the pattern of mobility and characters of vindoline, catharanthine, ajmalicine, vincristine (vcr) and serpentine (ser) present in the suspended cells of *C. roseus*. In HPLC analyses, 20 µl of cell extract was injected three times. Fig 2 explains the alkaloid content present in the suspension media. Certain important alkaloids could be obtained from the media itself without sacrificing the cells. In this study callus induction was observed in MS media after 6 weeks on supplementing the hormones. Vermicompost extract medium alone gave cent percent (Table 1) response with respect to callus formation and development of roots and shoots from the callus within 3-4 weeks. Similarly on spraying of coelomic fluid (vermiwash) also resulted in 90% callus development and in both cases the developed callus could be sub cultured for four times. The earlier reports have shown that with combination of different hormones there was only 70 % survival (Haq *et al.,* 2013; Kalidass *et al.,* 2010) and that auxins- 2, 4-D supported the growth of callus culture.

Table 2. Results of the standard alkaloids detected and their Retardation factor (Rf)

Alkaloid	R_f
Serpentine	0.1
Vincristine	0.25
Vinblastine	0.35
Cataranthine	0.75
Vindoline	0.85
Ajmalicine	0.95

Plate 1. Callus formation from leaf explant of *C. roseus* on vermicompost extract medium without any supplementation

The production of mass callus from MS medium with 3 mg/L each of 2, 4-D and Kn was also reported (Taha *et al*., 2008). The importance of BAP in multiple shoot formation in *V. rosea* and other members of this family are widely reported (Ravindra *et al*., 2004; Richa *et al*., 2008). Cytokinins determine the regeneration response in explants and enhance the number of meristematic cells as well as the cell division rate. The nature of callus also varies with the change in growth regulator. During the i*n vitro* initiation of callus, cell differentiation and specialization that occur in parent plant is reserved and cells of explant become dedifferentiated. Many factors determine the ability of a specific tissue to form callus. The chemical factors include mineral nutrition and plant growth regulators, environmental factors, such as light, temperature and humidity. The present results reflect that explants obtained from different parts of the same plant behave differently in the same culture media. In this study there was callus development and tissue differentiation only in explants of leaf and no response from the nodal tissue. This is in accordance to the earlier reports regarding the growth regulators in earthworm castings containing gibberllins (GA3) 2.75µg/g, cytokinins (IBA) 1.05 µg/g and auxins (IAA) 3.80 µg/g (Abdellatef and Khalafallah, 2008; Grapeli *et al.,* 1985). The vermicompost is rich in vitamins, enzymes, antibiotics and growth hormones and hence provides balanced nutrients to the plant making them resistant against pests were as well reported (Atlavinyte and Vanagas, 1982). These reports strongly support the present investigation suggesting vermicompost and its extracts can support better growth and development of *in vitro* cultured plantlets without any chemical supplementation. Suspension culture was successfully developed using vermicompost extract and coelomic fluid in the 3:1 ratio. The suspension culture of *Catharanthus roseus* from stem and leaf explants on medium containing NAA and kinetin has been established (Zhao *et al.,* 2001). A number of nutritional factors as well as the growth factors 2,4-D and IAA were studied to determine their influence on growth and alkaloid formation in *C. roseus* suspension cultures (Hirata *et al.,*1990; Miura *et al.,*1987; Miura *et al.,* 1988; Scragg *et al.,*1988). Similar to many chemical supplements used by mentioned researchers the suspension culture could be developed using vermicompost extract and coelomic fluid.

The content of total phenols in extracts, expressed as gallic acid equivalents (GAE) reported per gram of dry extract, ranged between 8.18±1.61 to 21.46±2.39mg GAE/g. Total phenolic contents 3.2 to 8.5 GAE (g/100g per dry matter) and total flavonoid contents 1.8 to 5.4 CE (g/100 of per dry matter) was reported (Rasool *et al.,*2011). In the present study TPC was found to be less in *in vitro* callus when compared with *in vivo* (86.88±0.12 mg/g) plants and flavonoids were higher (1.74±0.15 mg/gram) in callus. Comparatively the *in vivo* plants have shown better phenolics and *in vitro* callus have shown significant flavonoids content. The presence of ajmalicine, serpentine, vinblastine, vindoline and vincristine in the experimented samples were reported (Azimi *et al.,* 2008). Similar bands having the same Rf values were obtained for the suspension cell extract and callus, roots and shoots in the developed cultures on vermicompost extract. HPLC analysis showed that the pattern of mobility and characters of vindoline, catharanthine, ajmalicine (Rt=11.301), vinblastine (Rt= 6.079), vincristine (Rt= 6.674) and serpentine (Rt=3.591) were similar to the earlier studies (Guttman *et al.,* 2004). Suspension media extract (Fig 2) considered for the HPLC analysis also showed the similar peaks found in the cell extract samples. Though there are reports on callus induction in MS media supplemented with auxins and cytokinins; there was callus induction and plantlet regeneration from callus on vermicompost extract with or without supplementing with vermiwash (coelomic fluid) spray. This substantiates the presence of all the essential requirements for cell differentiation in vermicompost extract to consider it as an efficient medium to be used for micropropagation. Similarly cell suspension cultures and suspension media can be considered for recovery of the alkaloids released into the

suspension media for pharmaceutical purpose for development of the drugs. The repeated development of suspension cultures provides the necessary essential compounds within shorter period of time. This will be an advantage over sacrificing the plants grown in natural habitats.

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