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Genetic relatidness and *In vitro* antimicrobial activities of alkaloids isolated from Indian varieties of *Catharanthus roseus* (L.)

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Abstract
Catharanthus roseus (L.) is one of the most intensively studied for its anticancerous property but phytopathogenic activity of the plant has not been much studied. Callus induction from *C. roseus* was optimized by varying various harmone concentrations and the best result was obtanined in MS medium fortified with 2mgL^{-1} 2, 4-D and 0.1 mgL^{-1} kinetin. Antimicrobial activity and minimum inhibitory concentration showed that the 20, 40, and 80% leaves, shoot, and root extract inhibits Gram positive *B. subtilis* and phytopathogenic fungus *F. oxysporium, A. niger, Rhizoctonia solani, Colletotrichum falcatum, Cylindrocladium scoparium* respectively. Genetic relatidness was determined by RAPD analysis revealed that, white and violet colored *C.roseus* flowered plant was closely related than rosy pink coloured flower.

Keywords: Vinca rosea, Antimicrobial activity, RAPD.

INTRODUCTION

Catharanthus roseus (L.) is a pubescent herb belonging to Apocynaceae family and indigenous to Madagascar. This plant is majorly cultivated in South East Aisa including South Africa, Caribbean Islands for medicial importance as well as an ornamental plant [19, 11]. They secrete majorly indole alkaloids (vincristine and vinblastine) which having oncolytic activity [6, 15] as well as inhibit the polymerization of tubulins [11], inhibitory to *Aedes aegyptiiis* [22]. However, alkaloids were extracted from wild varities and were secreted in a very low quantity. The continuous and non organized exploitation has resulted in many plants becoming rare some even becoming extinct, besides these plant collected as minor producer; show a wide disparity in their values [3]. Low yield of these alkaloids from plants and the increased demand for them combined with relatively high market price have prompted intense search for alternative source for these alkaloids such as synthesis, semi-synthesis [10], and cell and tissue culture [12]. The prospects of producing alkaloids from cell cultures have been reviewed earlier [23]. However vincristine and vinblastine have been detected in callus or organ cultures of *C. roseus* [7, 8]. A successful establishment of callus cultures depended on the discovery of IAA (indole3-acetic acid), the endogenous auxin [21, 25]. For callus induction and various development processes, several explant sources (root, stem, flower, and anther) were used on different media [1, 13]. Fast growing callus was induced on 2, 4-D (0.5-2.0 mg/l) fortified medium for almost every explants source. Callus obtained were soft, white and friable [13]. Hence the present work we tried to improve the media for culturing of *C. roseus* as well as elucidate their phytopathogenic activity and minimum inhibitory concentration against multiphytopathogens. Genetic relatidness of three coloured flower (white, violet and rosy pink) *C. roseus* plant by using RAPD markers were also evaluated.

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MATERIALS AND METHODS

Seeds were collected from different varietites of *C. roseus* (L.) plants (rosy pink, white and violet flower coloured) from local nursery of Nagpur, Maharastra, India. Seeds were surface sterlized with 0.1% $HgCl_2$ for 2-3 mins followed by rinsed with distilled water 3-4 times. Surface sterilization of explants (hypocotyls, leaves, node and internode) was carried out by rinshed with distilled water followed by 0.1% $HgCl_2$ for 1 min.

Media employed and Effect of plant harmone on seed germination

Callousing media contains Mussirage and Skoog media (basal media) supplemented with auxin and cytokinin either in mixture or separately at different concentrations v.i.z., 1, 2, 3 mgL^{-1} auxin and 0.05, 0.1, 0.2 mg L^{-1} , kinetin, 2.0 and 3mg L^{-1} BAP respectively, while Regeneration media contains Macro stock ($CaCl_2$-5gL^{-1}, FeEDTA- 344 mgL^{-1}) and Micro stock (sucrose 3g L^{-1}, Casein hydrolysate- 2g, Kinetin- 0.2mL, BAP-0.2mL, pH-5.8 Agar-0.82g) respectively. Effect of gibberlic acid at different concentrations (0.02, 0.04, and 0.06%) on the germination of seed as well as germination capacity of different parts of plant/plantlets (hypocotyls, leaves, nodes, and internodes) was also observed.

Callus and Suspension Culturing

The callus was sub-cultured at every 4-6 weeks on fresh slants of MS media containing 4 mgL^{-1} 2, 4-D and 0.1 mgL^{-1} BAP. Suspension culturing of callus were carried out by constant shaking of callus (MS broth) under aseptic condition in on rotatory shaker at

100- 250 rpm to facilitate aeration and dissociation of cell clumps into small pieces. The flask was further kept on an orbital shaker (@110rpm) at temperature 24°C.

Thin layer chromatography and Secondary metabolite detection

Alkaloids are extracted from plants parts (root, leaves and stem) into a weak acid alcoholic solvent (10% acetic acid in ethanol) followed by precipitated with conc NaOH and crude extracts was tested for the presence of alkaloids. Dried plant parts were soaked in alcoholic solvent (10% acetic acid in ethanol) for 24h followed by crushed in the pestle mortar along with weak acid. The acid was filtered out by using muslin cloth. The extract was concentrated by heating on water bath and precipitated by NH_4OH. The precipitate was washed with 1% NH_4OH and dissolved in ethanol. The alkaloid was detected by thin layer chromatography by using methanol: conc NH_4OH (200:3) solvent system. Alkaloids were detected by spraying air dried plates with dragendroff reagent and change of colour was recorded.

***In vitro* antimicrobial assay**

The different varieties of catheranthus roseus (L.) plant were used for evaluating antimicrobial effect on the test phytopathogens including Gram-positive (B. subtilis, M. luteus and S. aureus) and Gramnegative bacteria (E. coli, K. pneumoniae, P. vulgaris and P. aeruginosa) and phytopathogenic fungi i.e., A. flavus, A. niger, Rhizoctonia solani, Fusarium oxysporium, Macrophomina phaseolina, Colletotrichum falcatum, and Cylindrocladium scoparium respectively. Fresh plant parts (leaves, roots, stem) were separately washed with distilled water and finally with sterile water. They were then crushed and ground in a pestle mortar seperately by adding sterile water (@1:1 w/v) and filtered through cheese cloth followed by centrifuged at 5000 rpm for 20 min. supernatant colloected after centrifugation were taken as standard plant extract solution (100%). The boiled plant extract (60˚C) were added to sterilzed, melted NA/ PDA media at different levels. The amended medium was poured into sterilized petridisheds and the dishes were inoculated in laminar air flow chamber with test fungus by placing uniform of 9 mm diameter from 4 days old- culture. The diameter of the fungal colony was measured after 96hr. Three replications were maintained at the laboratory temperature (28±1˚C). The percent inhibition of growth of the test fungi was calculated by the formula [24]:

$$I = \frac{100 \times (C-T)}{C}$$

Where, I= percent inhibition of fungal growth; C= growth of control; T= growth in treatment

Minimum Inhibition Concentration (MIC)

The MIC of the different plant part extract (leaves, stems and roots) against phytopathogens was determined by serial dilution of extracts with distilled water, with a series of concentrations ranging from 20, 40, 60, 80 and 100%. The plant extract were added to NA/PDA media at different levels and phytopathogens were inoculated. Three replications were maintained at the laboratory temperature (28±1°C). MIC is the lowest concentration that completely inhibited visible fungal growth [14]. Each treatment was performed in triplicate.

Molecular characterizaion
G- DNA Isolation

Genomic DNA from *C. roseus* plant was extracted according to Doyle and Doyle [5] with little modification. 1.5g of plant tissue was weighed, quickly freezed in liquid nitrogen and grinded to a fine powder in a pestle and mortar, 75ml of extraction buffer was added in small aliquotes and grinded thoroughly. The homogenate was then transferred to a 250 ml flask.5ml of 20% SDS was added and mixed thoroughly using magnetic stirrer for 15-20 mins. Then the content was incubated at 65˚C for 10 min. 50ml of potassium acetate solution was added, mixed and incubated at 60˚C for 30 mins in order to precipitate proteins and polysaccharides. The precipitate was then removed by centrifugations at 25000g for 15 mins. To the supernatant six-tenth volume of isopropanol was added and allowed to stand at -20°C for at least 30 mins to precipitate DNA. DNAwas pelleted at 20000g for 15 mins.The supernatant was then drained off by inverting the tubes on filter paper for 2-3mins. DNA pellet was then redissolved in suspension buffer (add 3ml). 1.8ml isopropanol and 18ml 0.3M sodium acetate solution was added and allowed to stand at -20°C for 1 hr. DNA was repelleted by centrifugation and washed with ice-cold 80% ethanol and gently dried in vacuum or streaming nitrogen gas. The DNA pellet was redissolved in a suitable volume (0.5-5.0 mL) of TE buffer and purity and quantity were checked by spectrophotometeric analysis at 260 and 280 nm.

DNA Amplification Fingerprinting (RAPD)

RAPD amplification was carried out by using two decamer arbitary operon primers OPD8 (5'-GTGTGCCCCA-3') and OPD 12 (5'-CACCGTATCC-3'). The amplification was performed on Biorad thermal cycler with following cocktail: The 25-ml reaction mixture contained; 50 ng template DNA, 0.4mM dNTP, 25 pmoles of primer, 1 unit of Taq DNA polymerase (Genei, India), 10X reaction bu€er (10 mM Tris-HCl, 3 mM $MgCl_2$ and 30 mM KCl, pH 8.3). The amplification condition was revealed by for an initial 3 min denaturation at 94°C followed by 40 cycles of 1 min at 93°C, 3 min at 35°C and 2 min at 72°C. Amplified DNA fragments was analyzed by 2.5% horizontal agarose gel electrophoresis [17] and documented on a gel documentation system (Alpha-Imager, U.S.A).

DATA analysis

PCR reactions and electrophoresis were repeated at least twice to ascertain the reproducibility of the bands. Only reproducible bands were scored as present (1) or absent (0) in this study. These RAPD data, generated with ten primers, were used to compile a binary matrix for cluster analysis using the NTSYS-pc (version 1.6; Exeter Software, Setauket, N.Y.). Genetic similarity among accessions was calculated according to Jaccard's similarity coefficients [20] using the SIMQUAL (Similarity for Qualitative Data) routine. Jaccard's coefficients are defned as a/ (a+b+c), where a; the number of positive matches (i.e. bands common to two accessions), and b and c refer to the number of bands present only in accession 1 and 2, respectively [9]. The similarity coefficients were then used to construct a dendrogram using the UPGMA (unweighted pair-group method with arithmetical averages) through the SHAN (sequential, hierarchical, agglomerative and nested clustering) routine of the NTSYS-pc package software

RESULTS

In vitro seed germination of *C. roseus* (L.) takes 10-15 days while after gibbrellic acid treatment at different concentration, rapid germination was recorded within 48hrs. The totepotency capacity of the explants (nodes, internodes and hypocotyls) inoculated on the MS media containing Kinetin and BAP (2+2 mgL^{-1}) showed that, nodes and hypocotyl had regeneration ability while internodes fail to regenerates at all (Fig.1a, b).

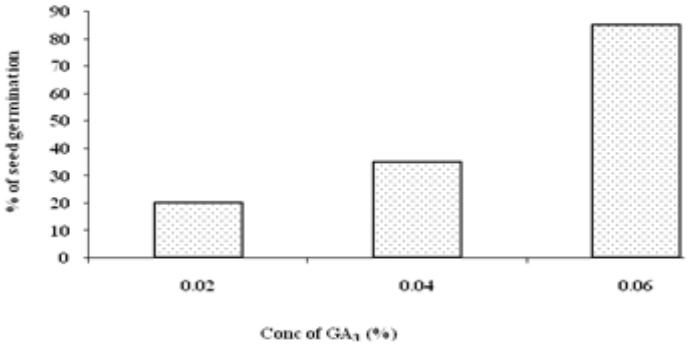


Fig 1 (A)

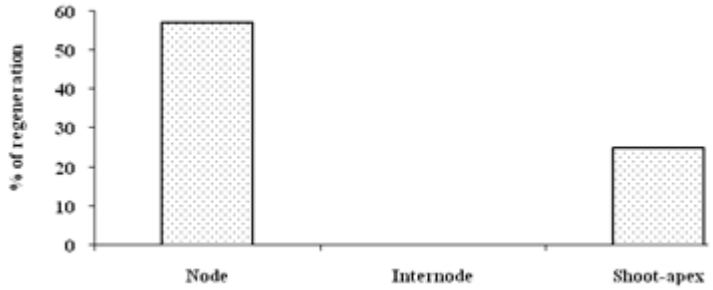


Fig 1 (B)

Fig1 (A): Effect of Gibberlic acid (different concentration) on seed germination of *C.* roseus; (B): Totepotency assay of different explant parts of *C. roseus*

Callus induction and suspension culturing

Effect of the induction of callus from *C. roseus* (L.) explants i.e., leaves and hypocotyls were inoculated in MS media containing different concentrations of auxin (2, 4-D) and kinetin and results were noted (Table 1). Suspension culture of callus was cultured on MS solid media containing 2mgL^{-1} 2, 4-D and 1mgL^{-1} BAP to proliferate the callus and establishment of suspension culture. Calli were then transferred to MS liquid media in a flask cotaining 2 mgL^{-1} 2, 4-D and 0.1 mgL^{-1} kinetin.The flask was kept on an orbital Shaker at a speed of 110 rpm and at a temperature of 24° C for increasing of biomass and alkaloid production. Alkaloid production was primilarily dectected by turning media into brown colour after two days, which indicates diffusion of secondary metabolites in media.

Table 1. Effect of MS media supplemented with various concentration of auxin and kinetin for callus induction.

Explant (No.)	Media used (MS-basal media supplemented with)		Callus induction	Time interval (days)
	2,4-D (mgL^{-1})	Kinetin (mgL^{-1})	No. of explant responded	
Leaves	1.0	-	1.0	8- 10
Hypocotyl	1.0	-	0.0	-
Leaves	2.0	-	7.0	6- 8
Hypocotyl	2.0	-	0.0	-
Leaves	3.0	-	1.0	8-10
Hypocotyl	3.0	-	0.0	-
Leaves	1.0	0.05	0.0	-
Leaves	2.0	0.05	3.0	8-12
Leaves	3.0	0.05	1.0	8-12

Antimicrobial activity

All the three varieties of *C. roseus* (L.) showed very promising antagonistic activities against multi-drug resistant pathogens. All the three varities showed antagonistic effect towards five important pathogenic fungi (F. oxysporium, A. niger, Rhizoctonia solani, Colletotrichum falcatum, Cylindrocladium scoparium) and Gram positive bacterium (B. subtilis, M.luteus) while Gram negative (E.coli) respectively (Table 2) while, it didn't showed any inhibition towards rest pathogenic microbes. Minimum inhibitory concentration experiment revealed that 20, 40, and 80% metabolite extracted from leaves, stem and root were able to inhibit above mentioned phytopathogens respectively.

Alkaloid detection

Paper chromatography and TLC were carried out for detection of alkaloids. Whatman paper and TLC plates were aliquoted with suspension culture extract and kept in TLC chamber with saturated methanol and ammonium hydroxide. After 30 minutes the plates and Whatman paper were sprayed with Dragendorff's reagent, they showed orange colour Fig 2.

Table 2. Antimicrobial activity of *C. roseus* on different phytopathogens.

Microorgainsms	*C. roseus* Plant Extract (100%)
B. subtilis	30±1.4
M. luteus	18±0.8
S. aureus	-
E. coli	20±2.2
K. pneumoniae	-
P. florescences	-
P. aeruginosa	12±0.5
F. oxysporium	20±3.1
Rhizoctonia solani	20±2.3
A. flavus	-
A. niger	35±2.2
Macrophomina phaseolina	-
C. scoparium	22±1.4
C. falcatum	15±3.0

Values represent average of five replications ± standard deviations.

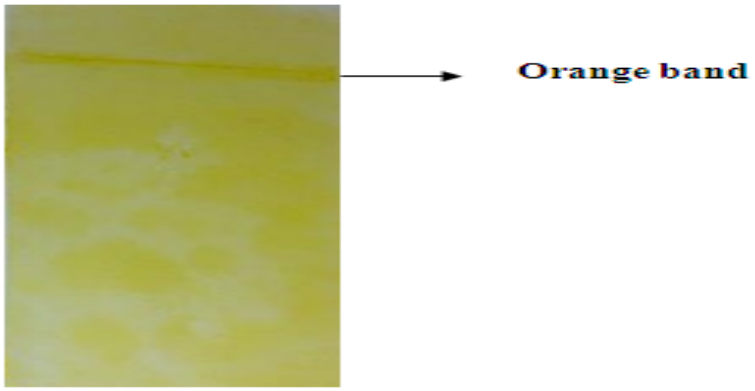
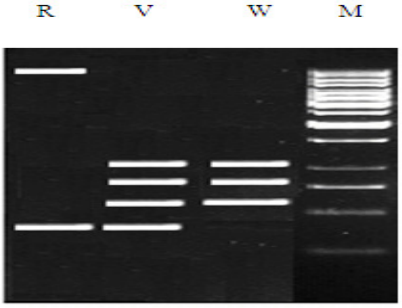


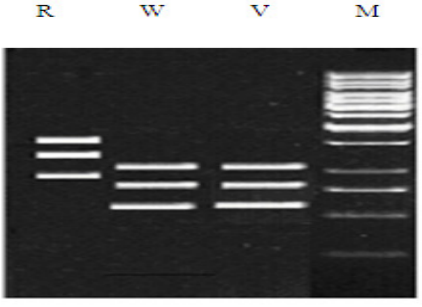
Fig 2. Detection of alkaloids (orange band) in in vitro cultured tissue

DNA Fingerprinting

DNA amplification experiment was conducted to distinguish and identify the different species of *C. roseus* (L.). The fingerprints were obtained using RAPD primer amplification was scored as presence and absence of bands and it was calculated using Nei's genetic distance among the species. Unique fingerprinting was obtained by using Primer OPD8 from three different coloured *C. roseus* plant i.e., white, violet and rosy pink flowered plants. White and violet has produced 3 fragments each with an approximate size of 1200 bp, 900 bp and 700 bp designated as OPD 1200, OPD900 and OPD700 (Fig. 3a, b). White and violet coloured flower plant showed similarity index value as 0.85 while rosy pink flower showed similarity value of 0.4. Primer OPD12 showed a total of 9 DNA fragments, white and violet flowered plant produced 3 monomorphic bands of approximate size of 900 bp, 700 bp, and 500 bp, designated as, OPD 900, OPD700, and OPD500. Similarity index between all the three species revealed that white flower showed similarity value of 0.92 with C.roseus violet while, C.roseus rosy pink coloured flower showed the similarity value of 0.36 (Table 3).

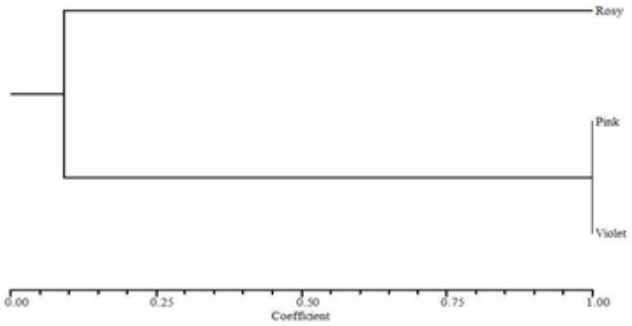


PCR amplified DNA fingerprinting of Catharanthus species with primer OPD-8. (R-rosy pink, V-violet, Wwhite flower of *C.roseus*)



PCR amplified DNA fingerprinting of Catharanthus species with primer OPD-12 (R- rosy pink, V-violet, Wwhite flower of C.roseus)

(A)



(B)

Fig 3. PCR amplified DNA fingerprinting of catharanthus species (A): with primer OPD-8. (R- rosy pink, V-violet, W- white flower of C.roseus) and OPD-12); (B): Genetic relatidness of different coloured flower *C. roseus* plant.

Table 3. Similarilty Index of different coloured flower (White, Violet and Rosy pink) based on RAPD marker amplification by using OPD-8 and OPD-12 primers.

C. roseus	White	Violet	Rosy pink
Similarity Index with OPD-8			
White	1.0	0.85	0
Violet	-	1.0	0.4
Rosy pink	-	-	1.0
Similarity Index with OPD-12			
White	1.0	1.0	0.33
Violet	-	1.0	0.33
Rosy pink	-	-	1.0
Combined Similarity Index			
White	1.0	0.92	0.2
Violet	-	1.0	0.36
Rosy pink	-	-	1.0

DISCUSSION

Natural flora contains several novel compounds including drugs. There are huge numbers of medicinal plants distributed all over the world. *C. roseus* is one of the most extensively studied for its anti-cancerous property. Among the important compounds identified, vincristine and vinblastine are most important for this deadly disease. The level of the active compounds in plant causes serious concern for nature preservation. Taking other limitations in views, plant cell culture methodologies have been applied. *C. roseus* has been extensively studied for its anticancerous activity. Till now alkaloids have been isolated, many are of high therapuetic values for curing human disease, but little emphases had been given in plant disease management [16]. Alkaloids are present in a very nominal amount in plant parts as well as their complex structure makes its chemical synthesis very tidious. The present experiment was carried out to resolve the problem, an effective in vitro culturing of the plant for the extraction of more amounts of metabolites as well as analyse their antimicrobial potential against many phytopathogens.

The seed germination of *C. roseus* (L.) normally takes 10-15 days for germination, but after treatment with gibberellic acid showed germination in 48 hrs and was directly propotional to the GA_3 concentration. It breaks the dormancy of the seeds and induces cell elongation. For callus, culturing mainly the hypocotyl and leaves as explants were selected because these regions contains more merestmatic tissue and would proliferate faster to form callus [1, 13]. Callus was obtained in MS media containing different concentration of auxin (1, 2 and 3 mgL^{-1}) and showed that best proliferation was obtained in the media containing 2 mgL^{-1} 2, 4-D and were also evident by Mujib et. al. [13] reports, that the callus grows hastens by appriation of 2, 4-D (0.5-2.0 mgL^{-1}) in the fortified medium. The callus obtained was transfered to the MS media without agar in a flask containing 2mgL^{-1} 2, 4-D and 0.1mgL^{-1} kinetin. After 48 hrs, callus turned into brown coloured showed that the secretion of some substances in the medium [4, 27]. The TLC plates and whatmann paper aliquoted with the extract, kept in the mobile phase in a TLC chamber (methanol + NH_4OH) for about 2 hrs when sprayed with dragendorff reagent showed orange colour which confirms the presence of alkaloids. Antimicrobial activity of *C. roseus* plant was also reported by many workers [16, 2, 18]. Minimum inhibitory concentration of plant extracts revealed that 20% leaves metabolite extract inhibits all phytopathogens, while root and shoot showed inhibition at 80 and 40% extract respectively. We concluded in this experiment that leaves produces/ accumulated more amounts of antimicrobial alkaloids than shoot and root respectively. Our result was also strenthgens by Sarvanan and Valluvaparidasan [18]. DNA fingerprinting technique had the major advancement in identification of species or measure of genetic distance [26]. In the present study DNA fingerprinting was used to produce unique fingerprinting, which would differentiate among closely related species. Our study revealed that the C.roseus white and violet coloured flower are simillar to each other while rosypink flowered plant falls under distinct clusters.

In Conclusion, the Callus induction was carried out with various hormone concentrations and best result was obtained in MS medium fortified with 2mgL^{-1} 2, 4-D and 2mgL^{-1} 2, 4-D and 0.2mgL^{-1}

kinetin. 20, 40, and 80% leaves, shoot and root extract of C.roseus plant showed antimicrobial activity against multiphytopathoges as evident in present study also showed by presence of alkaloids through TLC assay. Genetic relatidness of DNA fingerprinting was also studied and found that C.roseus white and violet were closely related than rosy pink flowered plant.

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