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# IN VITRO PROPAGATION AND ASSESSMENT OF GENETIC FIDELITY USING RAPD MARKERS IN CATHARANTHUS ROSEUS

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## ABSTRACT

*Field grown Catharanthus roseus axilary nodes, leaves and hypocotyl explants were aseptically cultured on MS basal medium fortified with different concentrations of cytokinins (BAP, Kinetin) and auxins (NAA, 2,4-D) along with sucrose and maltose as altered sugar source. The leaves and hypocotyls generated profuse compact mass of morphogenetic callus with different colours and textures on different media tested. Maximum number of shoots (83.34%) was observed on MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA in 13 days using axillary node. Hypocotyls cultured on MS medium consisting 0.25 mg/L BAP and 0.5 mg/L NAA produced higher percentage (93.86%) of callus which was comparable to leaf explants (94%) on MS medium containing 1.0 mg/L Kinetin and 1.0 mg/L 2,4-D. The proliferated callus when sub-cultured on MS basal medium fortified with 4.0 mg/L BAP and 1.5 mg/L NAA generated direct organogenesis within 25 days, which in turn generated leaf primordia in next 7-10 days. RAPD being universal marker were used to access the genetic stability in the organogenetically produced shoots using 12 RAPD primers and compared with original mother plant. Dendrogram was constructed using distance program which validate the genetic fidelity by showing 84% genetic relatedness to the mother plant.*

***Keywords: Catharanthus roseus, casein hydrolysate, organogenesis, propagation, somaclonal variation***

## I. INTRODUCTION

Genetic variations from cultured tissue explants have become a new method to select mutants that have pre-eminent characteristics for crop improvement. Somatic embryogenesis whether direct or indirect from callus resulted in higher degree of variants. Crop improvement programme mainly rely on somaclonal variations which affect genetic integrity through micropropagation. The regenerants have genetic characteristics that differ from donor mother plant of the cultured tissue. The use of increased concentrations of growth hormones and time duration is a major factor contributing to genetic variations. Elevation in frequency of regeneration correlates to increase in abnormal growth which might be the result of epigenetic variations. Our present investigation reports morpho-genetically dissimilar shoot regeneration still showing 84% relatedness to the mother plant. *Catharanthus roseus* is known as the common of Madagascar periwinkle, though its name and classification may be contradictory in some literature because this plant was formerly classified as the species *Vinca rosea*, *Lochnera rosea* and *Ammocallis rosea. C. roseus* is a perennial, evergreen herb in the dogbane family (*Apocynaceae*) that was originally native to the island of Madagascar. It is abundantly naturalized in many

regions, particularly in arid coastal locations. It is grown commercially for its medicinal uses in Australia, Africa, India and Southern Europe and cultivated as an ornamental plant almost throughout the tropical and subtropical world. It is cultivated mainly for its alkaloids vinblastin and vincristine, which possess anticancerous activities[1]. *C. roseus* contains known antibacterial, antifungal, antidiabetic, anticancer and antiviral activities. The *Catharanthus* (or Vinca) alkaloids comprise a group of about 130 terpenoid indole alkaloids (TIAs). Many TIAs are true lead compounds for drug development, including two commercially important cytotoxic dimeric alkaloids, vinblastine and vincristine. These are utilized in a number of different cancer chemotherapies. Due to the pharmaceutical importance and the low yield in vinblastine and the related alkaloid vincristine, *C. roseus* became one of the best-studied medicinal plants. At present, only precursors and semi-synthetic derivatives of vinblastine and vincristine are obtained by chemosynthesis, cell and hairy root cultures[2]. Accordingly, it is feasible to establish a genetic engineering method for *C. roseus*, aiming at higher production levels of these low yield alkaloids.

In *C. roseus*, plant regeneration has been reported from callus initiated from a range of explant material; for example, mature zygotic embryo, hypocotyl, cotyledon, leaf petiole, stem node, shoot tip and immature zygotic embryo[3,4]. However, in most of these reports, the formation of shoots and plantlets in *C. roseus* is rather infrequent, transient and requires long duration. In addition, all of the reports about somatic embryogenesis of *C. roseus* published so far have either unstable regeneration from any type of explant or lack the corroboration of morphology and histology, which make genetic transformation studies on *C. roseus* difficult[5]. Although the plant has already been exploited for its medicinal aspects but regarding its extinction and existence in threatened species, its mass multiplication is a prerequisite for incoming years. Plant cell tissue culture has offered a very novel technique to mass multiply, true to type and providing disease resistant plants in controlled conditions[6,7,8,9,10].Although direct regeneration have been observed in *Rauwolfia serpentine*, a member of *Apocyanaceae* family but this is first report of direct regeneration without embryo formation from callus phase and stability of genetic fidelity in *C. roseus* using leaf segments.

## II. MATERIALS AND METHODS

### 2.1 Plant Material

*C. roseus* seedlings used for callus induction, shoot regeneration, direct organogenesis and analysis of somaclonal variation were procured from the Botany Department of Kurukshetra University, Kurukshetra, Haryana, India.

### 2.2 Explant Preparation

The disease free young leaves, hypocotyls, axillary nodes were collected from 4 month old healthy plants. The explants were excised from the plant and the contaminants were washed under running tap water for 45 min. Different explants were taken for culture and washed in the liquid detergent Tween-20 (few drops per 100 ml solution) and then rinsed in running tap water. The explants were surface sterilized with standard aqueous 0.1% mercuric chloride solution in laminar air flow. Different treatments of 2-5 min were given to optimize the sterilization procedure and then rinsed 4-5 times with sterilized distilled water. After trimming the cut ends, surface sterilized explants were cultured on the culture medium.

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### 2.3 Media and Callus Initiation

The sterilized leaf and hypocotyls were cultured on medium supplemented with various auxins viz. 2,4-dichlorophenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA), indole-3 acetic acid (IAA) and cytokinins viz. 6-benzyladenine (BAP) and kinetin (KN) alone or in different combination for callus initiation and proliferation[11]. Eight different callus induction media (Table 1) were used which consisted of a combination of Kinetin as cytokinin and 2,4-D as auxin. In the beginning kinetin concentration was kept constant (0.5 mg/L), while 2,4-D concentration was raised from 0.25 mg/L to 1.0 mg/L. In further medium, 2,4-D concentration was

**Table 1-Percent callus induction obtained from hypocotyls explant of *C. roseus***

| Medium | Total number of explants | Number of experiments | Medium code | Percent callus induction | Mean ± S.E. |
|---|---|---|---|---|---|
| **MS + 0.5 mg/L KIN + 0.25 mg/L 2,4-D** | 45 | 1 | CM1 | 25.3 | 26.7 ± 3.15 |
| | | 2 | | 29.8 | |
| | | 3 | | 25 | |
| **MS + 0.25 mg/L KIN + 0.5 mg/L 2,4-D** | 48 | 1 | CM2 | 23 | 25.8 ± 3.42 |
| | | 2 | | 24.5 | |
| | | 3 | | 30 | |
| **MS + 0.5 mg/L KIN + 0.5 mg/L 2,4-D** | 45 | 1 | CM3 | 55 | 56.5 ± 4.39 |
| | | 2 | | 58.5 | |
| | | 3 | | 56 | |
| **MS + 1.0 mg/L KIN + 0.5 mg/L 2,4-D** | 48 | 1 | CM4 | 57 | 57.5 ± 4.38 |
| | | 2 | | 57.5 | |
| | | 3 | | 58 | |
| **MS + 0.5 mg/L KIN + 1.0 mg/L 2,4-D** | 48 | 1 | CM5 | 60 | 57.7 ± 4.54 |
| | | 2 | | 58.3 | |
| | | 3 | | 55 | |
| **MS + 1.0 mg/L KIN + 1.0 mg/L 2,4-D** | 48 | 1 | CM6 | 94 | 93.86 ± 5.63 |
| | | 2 | | 95.6 | |
| | | 3 | | 92 | |
| **MS + 0.5 mg/L BAP + 0.25 mg/L NAA** | 45 | 1 | CM7 | 93 | 93.16 ± 5.58 |
| | | 2 | | 94.5 | |
| | | 3 | | 92 | |
| **MS + 0.25 mg/L BAP + 0.5 mg/L NAA** | 45 | 1 | CM8 | 90 | 90.6 ± 5.52 |
| | | 2 | | 92 | |
| | | 3 | | 90 | |

**Abbreviation:**

**S.E. = Standard Error**

kept constant (0.5mg/L), while BAP concentration was raised from 0.5 to 1.0 mg/L. MS basal medium was further supplemented with BAP (0.5 mg/L) and NAA (0.25 mg/L), while the concentrations were interchanged consisted of BAP (0.25 mg/L) and NAA (0.5 mg/L). The cultures were incubated under cool fluorescent lights with 1500-2000 lux for 16 hr at a temperature of 26±2°C and 80% relative humidity. Data were recorded up to seven weeks of culture.

### 2.4 Callus Induction

The callus induced from different explants were transferred on MS basal medium supplemented with different concentrations of plant growth regulators consisted of combinations of different concentrations of 2,4-D, Kinetin, BAP and NAA. In further sugar source was replaced with 3% maltose and dextrose in place of sucrose. BAP concentration was raised from 1.5 mg/L to 4.0 mg/L whereas NAA concentration was kept constant at 1.0 mg/L and 1.5 mg/L. Callus proliferated was cultured on two different media containing BAP (3.0 mg/L) and NAA (1.5 mg/L) and medium containing BAP (  mg/L) and NAA (1.5mg/L). After 25 days, proliferated callus was subcultured on medium containing BAP (4.0 mg/L) and NAA (1.5 mg/L). The regenerated calli were further subcultured for root formation on half strength MS medium containing 0.2mg/L IAA.

### 2.5 Shoot Proliferation

Axillary nodes were excised from the young seedlings grown *in vivo* conditions and were cultured on eight different shoot proliferation media (Table 2) consisted of different concentration and combination of BAP and NAA as plant growth regulator. The BAP concentration was raised from 0.5 mg/L to 2.0 mg/L, while NAA in combination was kept constant (1.0 mg/L). Axillary node explants were implanted vertically. Test tubes and flask containing the explants were sealed with sterilized cotton plugs and incubated for 4 weeks at 25±2°C under a 16 hour photoperiod. Radiation source was supplied by soft white fluorescence tubes. Percent shoot proliferation and multiple shoot formation was recorded after four weeks of culture.

### 2.6 Genomic DNA Isolation

Genomic DNA was isolated from young leaves of regenerated shoots *in vitro* and seed propagated mother plant grown in greenhouse using cetyltrimethyl ammonium bromide (CTAB) extraction method with minor modifications[12,13]. Around 500mg of shoots from selected micropropagated plant was ground to a fine powder in liquid nitrogen and then incubated at 55°C for 25 min in 5-8 ml of CTAB extraction buffer containing 1.0% (w/v) CTAB, 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 5 mM EDTA, 100 mM Tris-HCl, (pH 8.0). The suspension was then extracted twice with an equal amount of chloroform: isoamylalcohol (24:1) and centrifuged at 6000g for 10 min. The resulting upper aqueous phase was recovered and precipitated with 2/3 volumes of chilled isopropanol. The flocculent white precipitate observed was retrieved via centrifugation at 5000g for 5 min. Pellet was washed with wash buffer (76% ethanol, 10mM ammonium acetate) for 20 min after discarding the supernatant followed by another centrifugation at 2000g for 3 min. The wash buffer was decanted and pellet allowed to air dry at room temperature after which it was resuspended in 1 ml TE solution. Ribonuclease was added to a final concentration of 10 ug/ml and incubated at 37°C for 30 min. DNA was then precipitated in 2.5

volumes of cold ethanol, centrifuged at 10,000g at 4°C for 10 min, pellet air dried and resuspended in 1 ml TE solution.

### 2.7 RAPD Analysis

Genetic fidelity between mother plant and *in vitro* regenerated plant was assessed using PCR based RAPD markers. Twelve random oligonucleotide primers (Table 3) were selected from RAPD 10-mer primers (Axygen Biotech Pvt Ltd) in 20 ul PCR reactions mixture. Each PCR reaction mixture contained 0.5 ul 10 mM dNTPs, 1 ul 10X Taq buffer, 0.5 mM of each dNTP, 50 ng of template DNA and 1.5U of Taq polymerase (Axygen Biotech Pvt Ltd). The reaction mixture was placed in gradient thermal PCR machine with following cycles: at 94°C for 5 min for first cycle, followed by 36 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C, and the final cycle at 72°C for 5 min. PCR products from 12 different primers and micropropagated shoots were analyzed via electrophoresis on 1% agarose gel at 80 mV for 2 h. A 1Kb ladder (Axygen Biotech Pvt Ltd) was used as a marker. The gels were stained with ethidium bromide, visualized under UV light and photographed.

**Table 3-Random primers showing amplification**

| S. No. | Primer Code | Sequence | No. of Bands | | Mono-morphic Bands | Poly-morphic Bands | Percentage of mono-morphism |
|---|---|---|---|---|---|---|---|
| | | | In Mother Plant | In Propagate plant | | | |
| **1.** | 6800-021 | GGGAAGCGTC | 6 | 5 | 5 | 1 | 83.33 |
| **2.** | 6800-023 | AAGCGGCCCT | 15 | 6 | 6 | 6 | 40 |
| **3.** | 6800-028 | TTCGGCGATG | 3 | 3 | 3 | 0 | 100 |
| **4.** | 6800-034 | GGAGCTGACT | 13 | 11 | 11 | 2 | 84.62 |
| | **Total** | | **37** | **25** | **25** | **12** | **67.57** |

### 2.8 Construction of Dendrogram

The DNA profile from each gel photograph was analyzed using the NTSYS-pc software programme. A binary matrix was constructed by scoring for either band presence (1) or band absence (0). Faint bands were excluded for the analysis. Similarity indices were generated for each 12 primers after inputting the binary matrix into software using Jaccard matrix. The resulting similarity and distance matrices were then used to construct the phenograms using UPGMA programme of NTSYS-pc software.

### 2.9 Calculation of Genetic Distance

Genetic distance between all samples relative to the mother plant were calculated using the Ni and Li's method[14], the estimation of genetic distance between samples were determined for all the twelve RAPD primers and values were then averaged to give final result.

## III. RESULTS AND DISCUSSION

### 3.1Callus Initiation, Induction and Proliferation

Callus was initiated using leaf and hypocotyl explants of *C. roseus* on MS basal medium supplemented with different plant growth regulators consisting of kinetin and BAP as cytokinin and 2,4-D and NAA as auxin. Before culture inoculation, the explants were surface sterilized using standard mercuric chloride concentration of 0.1% for 3 min which served best for giving 75% sterilization. Initially for the leaf explants, the concentration of kinetin was kept constant (0.5 mg/L), while raising the concentration of 2,4-D (0.25 to 1.0 mg/L). Further the concentration of 2,4-D was kept constant (0.5 mg/L) and raising the concentration of BAP (0.5 to 1.0 mg/L). Maximum (96%) callus induction was observed on medium containing kinetin (1.0 mg/L) along with 2,4-D concentration of (1.0 mg/L) in 18 days of inoculation initiating from cut ends. (Fig. 1). The percentage of callus induction reduced (35.86%) on lowering the concentration of BAP to half and 2,4-D to one fourth so concentration of plant growth regulators play a remarkable role in callus induction. The medium rest than above described consisted of a combination of BAP (0.25, 0.5 mg/L) along with NAA (0.25, 0.5 mg/L) in shown combination generated negligible results, or say no callus initiation was observed. In overall the combination of kinetin along with 2,4-D proves to be crucial for induction of green colored, brittle callus from leaf explants as compared to BAP and NAA as growth regulators supported by findings of earlier reports[15,16,17,18,19]. The hypocotyls cut into 1-2cm size were sterilized following standard procedure using 0.1% mercuric chloride for three minutes and were cultured on same combinations of growth regulators which were used for leaf explants, consisting of kinetin and 2,4-D at different concentration, along with BAP and NAA as auxins and cytokinins.

Maximum (93.86%) callus induction was observed on medium supplemented with BAP (0.5 mg/L) and NAA (0.25 mg/L) which was quite comparable (93.16%) on another medium consisting of kinetin (1.0 mg/L) and BAP (1.0 mg/L). The hypocotyls resulted in green color callus in lesser days (14-16 days) as compared to leaf (21-28days), while as compared the plant growth regulators consisting of kinetin (0.25 mg/L) along with 2,4-D (0.5 mg/L) generated least percentage of callus (25.8%). Moreover, the color of callus turned pale yellow. The results totally contradict the induction on the basis of explants and growth regulators supplemented. The combination of kinetin with 2,4-D well suited for generating a stimulus for callus induction using leaf explants but produced yellow color callus when hypocotyls were used. Moreover, the number of days taken to induce callus lessen by one week in case of hypocotyls as compared to leaf explants. It is reported that the proficiency of different concentration of 2,4-D (0.10, 0.25, 0.5, 1.0, 2.0, 5.0 mg/L) on callus induction which was significantly higher using nodal explants than leaf, but the callus obtained was pale yellow in color similar to

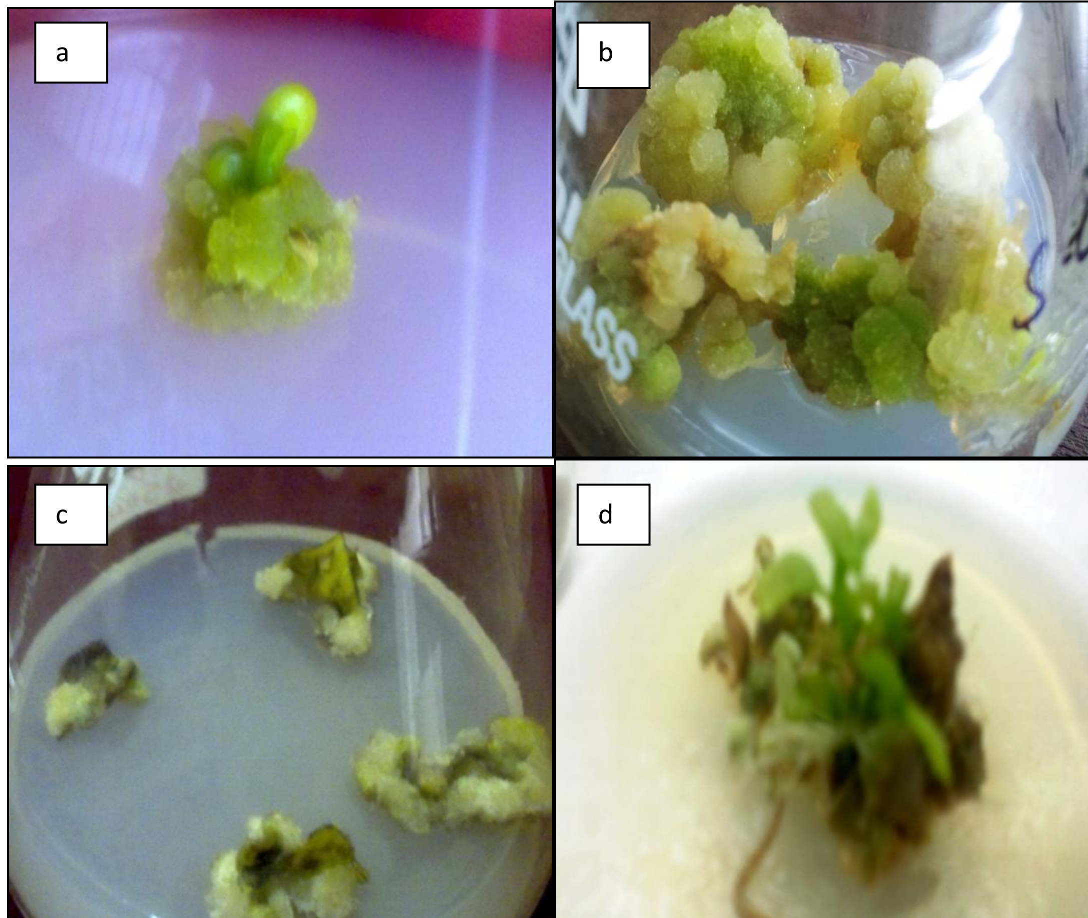


Fig. 1. Callus initiation from leaf explants cultured on MS medium fortified with 1.0 mg/L kinetin and 1.0 mg/L 2,4-D. b) Globular lustrous callus obtained from leaf explants cultured on MS medium fortified with 2.0 mg/L BAP only. c) Direct regeneration of leaf primordia from callus cultured on MS medium supplemented with 4.0 mg/L BAP along with 1.5 mg/L NAA without intermittent embryo stage. d) Direct organogenesis of cotyledon and roots from callus cultured on BAP (4.0 mg/L) and NAA (1.0 mg/L) along with 1.0 mg/L IAA.

our findings[20]. It is also observed that best deep green callus which turned into friable callus using internode with 2,4-D at a lower concentration (1.0 to 2.0 mg/L) while proliferation in combination with kinetin (1.0 to 5.0 mg/L) resulted in callus induction merely in 20-25 days[21]. Similar combination of BAP and NAA/IAA resulted in profuse mass of callus as reported by many scientist in various medicinal plants. The calli induced or initiated on different medium was subcultured on eight different medium having different combinations of kinetin with 2,4-D and BAP with NAA. A combination of 2,4-D better responded to mass proliferation of callus using leaf explants compared to BAP and NAA. So, concentration of 2,4-D and kinetin was raised to 2.0 mg/L and was further supplemented with casein hydrolysate and a different source of sugar i.e. 3% maltose. In a study, it is reported that MS medium supplemented with 2,4-D and Kinetin and half concentration of $NH_4NO_3$ led to induction of embryogenic callus while media supplemented with $KNO_3$ results in embryoid induction[22]. The proembryoid development can be enhanced by transferring callus to media lacking $NH_4NO_3$ and containing double the amount of $KNO_3$ as reported[23]. Casein hydrolysate gave black coloured callus in one week of culture but addition of maltose separately produced yellow color callus in merely 10 days. While hypocotyls raised calli were transferred to medium consisted of a combination of BAP (1.5 to 3.0 mg/L) and NAA (1.0 to

1.5 mg/L) the callus proliferated into lustrous green, compact and globular mass having shiny appearance. A raised concentration of BAP and NAA induce stimulus for mass proliferation of callus which in turn is a pre requisite for any tissue culture technique. The culture differentiated with high frequency through organogenesis into multiple shoots. It is also reported that leaf segments and young leaves when cultured on MS medium supplemented with Zeatin and IAA along with casein hydrolysate (500 mg/L) formed embryogenic callus[24].

### 3.2 Embryogenic Callus Potential

Callus initiated from hypocotyl and leaf explants using eight different media turned brown when subcultured on medium containing Kinetin (2.0 mg/L) and 2,4-D (2.0 mg/L) after 9 to 10 days. The callus showed embryogenic potential on two media one specifically containing BAP (2.0 mg/L) and other containing BAP (2.0 mg/L) and NAA (1.0 mg/L). The embryogenic calli showed shiny globular structures, while non-embryogenic callus was dull. The calli were further subcultured on medium containing BAP (4.0 mg/L) and NAA (1.5 mg/L) resulted in direct organogenesis of leaf primordial and roots in 30 days which was morphogenetically very fragile in nature as compared to mother plant. It was further reported that single node stem explants cultured on higher concentration of BAP (5.0 mg/L) with NAA (0.2 mg/L) produced regenerated shoots in gurmar (*Gymnema sylvestre* ) which when inoculated on auxin free half strength MS basal medium resulted in root primordial emergence from shoot base[25]. Another study observed direct organogenesis using hypocotyl explants in *Psoralea corylifolia* L. using BAP (3.0 uM) in combination with NAA (1.0 uM) very well similar to our findings[26]. In total 8-12 roots were observed on half strength MS medium supplemented with IAA (0.2 mg/L). The regenerated plantlets were unable to give rise to full plants. The Plants were used to check the clonal fidelity by isolating DNA and compared with mother plant.

### 3.3 Shoot Proliferation

The axillary nodes excised from *in vivo* grown young seedlings were sterilized using standard mercuric chloride solution at 0.1% concentration for 3 min which was sufficient to obtain 100% sterlization. The sterilized explants were cultured on different media (Table 4) consisting of different concentration of BAP and NAA as plant growth regulator. The proliferation of shoots from cultured explant was remarkably influenced by the type of concentration of the growth regulator used. Axillary node cultured on MS medium supplemented with different concentration of BAP alone showed the best results. The highest shoot proliferation (83.4 %) was observed on medium containing BAP (2.0 mg/L) along with NAA (1.0 mg/L) in 13 days, while least percentage (31.93%) of shoot proliferation was obtained on medium containing BAP (0.5 mg/L) and Kinetin (1.0 mg/L).The percent of shoot proliferation on medium containing BAP (0.5 mg/L) and NAA (1.0 mg/L), medium

**Table 3-Percent shoot proliferation from axillary node of *C. roseus* cultured on different media**

| Medium | Total number of shoots cultured (3 replicates) | Percent callus induction | Mean ± S.E. | Medium code |
|---|---|---|---|---|
| **MS + 0.5 mg/L BAP + 1.0 mg/L NAA** | 24 | 62.50<br>66.67<br>50.00 | 59.72 ± 2.925 | CMM1 |

| | | | | |
|---|---|---|---|---|
| **MS + 1.0 mg/L BAP + 0.5 mg/L NAA** | 24 | 60.00<br>57.14<br>54.55 | 57.23 ± 0.905 | CMM2 |
| **MS + 1.5 mg/L BAP + 1.0 mg/L NAA** | 24 | 75.00<br>87.50<br>62.50 | 75.00 ± 4.931 | CMM3 |
| **MS + 2.0 mg/L BAP +1.0 mg/L NAA** | 15 | 91.50<br>83.34<br>75.67 | 83.34 ± 9.127 | CMM4 |
| **MS + 1.0 mg/L BAP** | 26 | 61.53<br>55.56<br>75.00 | 64.03 ± 3.498 | CMM5 |
| **MS + 0.5 mg/L BAP** | 15 | 40.00<br>60.00<br>80.00 | 60.00 ± 6.991 | CMM6 |
| **MS + 0.5 mg/L BAP + 1.0 mg/L KIN** | 15 | 37.50<br>33.34<br>25.00 | 31.93 ± 2.286 | CMM7 |
| **MS + 1.0 mg/L BAP + 0.5 mg/L KIN** | 15 | 40.00<br>32.50<br>27.50 | 33.34 ± 4.223 | CMM8 |

containing BAP (1.0 mg/L) and NAA (0.5 mg/L) and medium containing BAP (0.5 mg/L) was almost comparable (59.72%, 57.23% and 60%) respectively. Average percent of shoot proliferation (75%) was obtained on medium containing BAP (1.5 mg/L) and NAA (1.0 mg/L). The percent of shoot proliferation as observed on eight different media varied from 31.93% to 83.34% . Multiple shoots were not observed on any of the media used only one or two shoots were observed otherwise the shoots just proliferated to 2-4 cm in 15 days. Similar findings were observed in *Vitex negundo,* where MS medium supplemented with BAP individually enhanced the induction of multiple shoots within an average of 8 to 12 days, while BAP (2.0mg/L) in combination with NAA (0.5 mg/L) resulted in a higher percentage (93%) of multiple shoot formation in *V. Negundo*[27]. The results were further supported in many other medicinal plants including *Gloriosa superba*, *Rauvolfia serpentine* and *Boerrhavia diffusa.*

### 3.4 RAPD Analysis

RAPD being universal markers were used to assess genetic stability of micropropagated plantlet[28,29]. The healthy plantlet was selected from a clonal collection of shoots that originally obtained from a single mother plant. Twelve random decamer oligonucleotide primers (Genaxy Scientific Pvt. Ltd, India) having high percentage of G+C content were used to check the fidelity of *in vitro* raised clones compared with mother plant of *C. roseus* for detecting somaclonal variations. Out of 12 primers screened, 4 primers generated unique set of amplified products (Fig. 2). Total 37 clear and reproducible bands

were obtained with an average of 9.25± S.D. bands per primer. Out of which 12 bands were polymorphic and 25 were monomorphic. The DNA amplification generated an average of 67.57% monomorphism using RAPD primers. The small number of amplicons distributed over distinct regions of genome was not adequate for analysis of somaclonal variations. But comparable number of bands was scored in various medicinal plants by using PCR based marker system[30,31,32]. Although the clonally raised plant was more fragile and short stature as compared to mother plant, but still very low variation was detected. The monomorphic pattern concludes that *in vitro* raised clones maintained their genetic integrity.

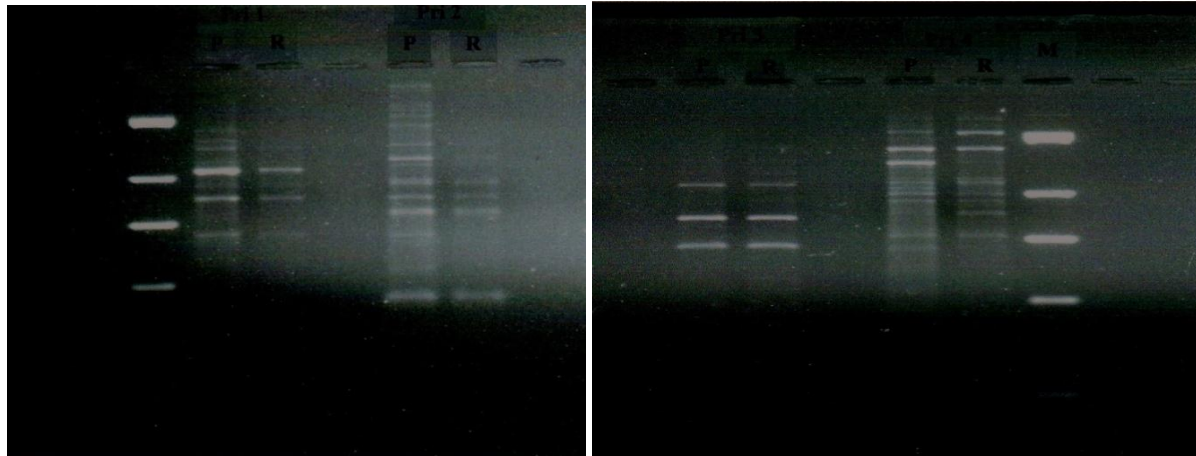

**Fig.2. RAPD profile generated using 4 RAPD decamer primers showing similar banding pattern in mother plant (P) and regenerant (R)**

### 3.5 Cluster Analysis

Statistical analysis of data obtained by NTSYS-pc analysis for each primer was scored on the basis of presence or absence of amplified products in the genotypes. Presence of band in one genotype was taken or denoted by one (1) and the absence of band represented as zero (0). Cluster analysis was performed on the basis of Jaccard similarity coefficient using RAPD data of 37 scored bands. Similarity value ranged from 0.676 to 1.0 according to RAPD analysis, micropropagated plant was grouped together with mother plant at similarity coefficient of 0.84. Our results well corroborate with that two another studies, which reported monomorphic amplified products using RAPD markers in *Alpinia galangal* and *Sapindus trifoliatus* revealed true to type nature of regenerated and mother plant[33,34]. It was suggested in earlier studies also that direct organogenesis, or direct organ formation, based on micropropagation system is much more stable genetically. The results revealed the significance of direct organogenesis of shoots in maintaining the regeneration frequency with morphogenetic competence. Out of 12 primers screened, 4 primers were found to produce clear reproducible bands and similarity index between mother plants and regenerant was found to be 0.676. The UPGMA dendrogram constructed based on similarity revealed 84% genetic relatedness between mother plant and regenerant which in turn validate the stability. In our research very low variations were observed between regenerant and mother plant which indicated that micropropagated *catharanthus* plant is genetically stable.

## IV. CONCLUSION

The *Catharanthus* (or Vinca) alkaloids comprise a group of about 130 terpenoid indole alkaloids (TIAs). Many TIAs are true lead compounds for drug development, including two commercially important cytotoxic dimeric alkaloids, vinblastine and vincristine. These are utilized in the developmet of hybrid drugs for different cancer chemotherapies. Due to the pharmaceutical importance and the low yield in vinblastine and the related alkaloid vincristine, *C. roseus* became one of the best-studied medicinal plants. At present, only precursors and semi-synthetic derivatives of vinblastine and vincristine are obtained by chemosynthesis, cell and hairy root cultures. So there is a prerequisite to establish a genetic engineering programme for *C. roseus*, aiming at higher production levels of these low yield alkaloids. In *C. roseus*, plant regeneration has been reported from callus initiated from a range of explant material; for example, mature zygotic embryo, hypocotyl, cotyledon, leaf petiole, stem node, shoot tip and immature zygotic embryo. However, in most of these reports, the formation of shoots and plantlets in *C. roseus* is rather infrequent, transient and requires long duration. In addition, all of the reports about somatic embryogenesis of *C. roseus* published so far have either unstable regeneration from any type of explant or lack the corroboration of morphology and histology, which make genetic transformation studies on *C. roseus* difficult. Plant cell tissue culture has offered a very novel technique to mass multiply, true to type and providing disease resistant plants in controlled conditions.

## V. ACKNOWLEDGEMENT

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