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Research Article

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Assessment of Variations in Different Cultivars of *Catharanthus roseus* by using Restriction Endonuclease and Rapd PCR

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ABSTRACT

Catharanthus roseus (L.) G. Don (family Apocynaceae) is an ornamental plant with medicinal values. It is an evergreen shrub and found to grow in the world in many places around the country. Alkaloids of this plant have a great medicinal importance to treat diabetes, malaria, cancer etc. Secondary metabolites interfere with the extraction of pure genomic DNA, therefore extracting DNA from Catharanthus roseus is a challenging task. This study describes a rapid and reliable cetyl trimethylammonium bromide (CTAB) protocol suited specifically for extracting DNA from plants which are rich in polysaccharides and secondary metabolites, and the protocol also excludes the use of expensive liquid nitrogen. Initially, the plants were grown as garden plants because of their beautiful flowers of different colors, such as pink, red and white. Though considerable variations can be observed in the species around the world, no informations on the origin is available. Attempts have not been made so far to study the genomic relations among Catharanthus roseus cultivars. In view of these facts the study was conducted for assessment of variations in different cultivars of Catharanthus roseus by using Restriction Endonuclease and RAPD PCR. In RAPD total seven primers were used out of which the amplifications of only three primers (OP-04, OP-06 and OP-07) were satisfactory. In Restriction Endonuclease, EcoRI and HindIII gave many bands in agarose gel. This work can lead to development of an efficient protocol to study the genetic diversity among different cultivars of Catharanthus roseus using Restriction Endonuclease and RAPD PCR.

Keywords: *Variations, Cultivars, Catharanthus roseus, Restriction Endonuclease, RAPD PCR.*

INTRODUCTION

Catharanthus roseus (L.) G. Don (family Apocynaceae) is an ornamental plant with medicinal values. It is an evergreen shrub and found to grow in the world in many places around the country. The genus *Catharanthus* is well reported for producing biologically active terpenoid indole alkaloids (TIAs) with over 130 compounds isolated and identified[1]. Alkaloids of this plant have a great medicinal importance to treat diabetes, malaria, anticancerous [2] etc. This plant is grown commercially for its medicinal and ornamental uses in India. The low yield of dimeric indole alkaloids from the plant (approximately 0.0005%) and their consequent high price have stimulated numerous efforts to develop alternative strategies for their production. As a promising alternative plant tissue culture technology has many advantages over traditional field cultivation and chemical synthesis, particularly for many natural compounds that are either derived from slow growing plants or difficult to be synthesized with chemical methods[3]. *Catharanthus* has *in vitro* cultured to make it available in large amount by various scientists [4, 5]. Initially, the plants were grown as garden plants because of their beautiful flowers of different colors, such as pink, red, white etc. Nowadays, new and improved verities of *Catharanthu* are also available. *Catharanthus roseus* is native to India and spread throughout the tropics and subtropics. Though considerable variations can be observed in gardens around the world, no informations on the origin is available. Attempts have not been made so far to study the genomic relations among *Catharanthus.*

In view of these facts the study was conducted for assessment of variations in different cultivars of *Catharanthus* by using Restriction Endonuclease and RAPD PCR. The polymerase chain reaction (PCR) based on molecular markers such as random-amplified polymorphic DNA (RAPD) and Restriction Endonuclease are being extensively used to study the genetic diversity in a number of plant species as well as cultivar level. To date, no report is available on applications of molecular markers in studies on the genetic diversity of different *Catharanthus roseus* varieties. This work can lead to development of an efficient protocol to study the genetic diversity among different cultivars of *Catharanthus roseus* using Restriction Endonuclease and RAPD PCR.

MATERIALS AND METHODS

Plant Material

The study involved two cultivars of *Catharanthus roseus* differing in petal color and shape grown naturally in the departmental garden of Botany, Patna University, Patna-5 (Table 1 and Fig. 1). Detections of different cultivars were based on the petal color and shape[6] in other species. Flower petal was separated as colorless (white) and colored (pink). Leaf samples of each genotype were collected from plants in three locations (populations). Three plants of each genotype were selected in each location based on morphological homogeneity.

Fig. 1: Morphological features of two *Catharanthus roseus* cultivars (Crw- *Catharanthus roseus* white and Crp - *Catharanthus roseus* pink)

Table 1: Morphological characteristics of two cultivars of *Catharanthus roseus* (L.) G. Don (family Apocynaceae)

Cultivars	Diameter and place of flower	Petal color	Stem-Leaf	Flower	Fruit
Crw	Inflorescence terminal, but apparently lateral, 1–2-flowered. Flowers bisexual, 5-merous, regular, almost sessile; sepals slightly fused at base, (2–)3–5 mm long, erect, green; corolla tube cylindrical, 2–3 cm long, widening near the top at the insertion of the stamens, laxly shortly hairy to glabrous outside, with a ring of hairs in the throat and another lower down the tube, greenish, lobes broadly obovate, 1–2(–3) cm long, apex mucronate, glabrous, spreading, white ; stamens inserted just below the corolla throat, included, filaments very short; ovary superior, consisting of 2 very narrowly oblong carpel, style slender, 15–23 mm long, with a cylindrical pistil head provided at base with a reflexed transparent frill and with rings of woolly hairs at base and apex, stigma glabrous.	White	Erect or decumbent, deciduous under shrub up to 1 m tall, usually with white latex; roots up to 70 cm long; stems narrowly winged, green, shortly hairy to glabrous, often woody at base. Leaves decussately opposite, simple and entire; stipules 2–4 at each side of the leaf base; petiole 3–11 mm long, green or red; blade elliptical to obovate or narrowly obovate, 2.5–8.5 cm × 1–4 cm, base cuneate, apex obtuse or acute with a mucronate tip, herbaceous to thinly leathery, glossy green above and pale green below, sparsely shortly hairy to glabrous on both sides.	unpleasant smell	Fruit composed of 2 free cylindrical follicles 2–4.5 cm long, striate, laxly shortly hairy to glabrous, green, dehiscent, 10–20-seeded. Seeds oblong, 2–3 mm long, grooved at one side, black. Seedling with epi geal germination.
Crp	Inflorescence terminal, but apparently lateral, 1–2-flowered. Flowers bisexual, 5-merous, regular, almost sessile; sepals slightly fused at base, (2–)3–5 mm long, erect, green; corolla tube cylindrical, 2–3 cm long, widening near the top at the insertion of the stamens, laxly shortly hairy to glabrous outside, with a ring of hairs in the throat and another lower down the tube, greenish, lobes broadly obovate, 1–2(–3) cm long, apex mucronate, glabrous, spreading, pink ; stamens inserted just below the corolla throat, included, filaments very short; ovary superior, consisting of 2 very narrowly oblong carpels, style slender, 15–23 mm long, with a cylindrical pistil head provided at base with a reflexed transparent frill and with rings of woolly hairs at base and apex, stigma glabrous.	Pink	Erect or decumbent, deciduous under shrub up to 1 m tall, usually with white latex ; roots up to 70 cm long; stems narrowly winged, red, shortly hairy to glabrous, often woody at base. Leaves decussately opposite, simple and entire; stipules 2–4 at each side of the leaf base; petiole 3–11 mm long, green or red; blade elliptical to obovate or narrowly obovate, 2.5–8.5 cm × 1–4 cm, base cuneate, apex obtuse or acute with a mucronate tip, herbaceous to thinly leathery, glossy green above and pale green below, sparsely shortly hairy to glabrous on both sides.	unpleasant smell	The dehiscent fruit consists of a pair of follicles each measuring about 25 mm in length and 2.3 mm in diameter, containing up to thirty linearly arranged seeds with a thin black tegument. On maturity, the follicles split along the length dehiscing the seeds.

Crw- *Catharanthus roseus* white and Crp- *Catharanthus roseus* pin

Isolation of genomic DNA

Genomic DNA was isolated from tender young leaves by the standard CTAB (Cetyl trimethyl ammonium bromide) method[7] with slight modifications. RNA was removed by RNaseA treatment. The DNA concentration was determined by comparison with a known concentration of lambda phase DNA[8]. The DNA quality as well as quantity was also checked by agarose gel and an UV-VIS Spectrophotometer (U.V Mini 1240). After quantification, the DNA was diluted with TE buffer (Tris 10mM and EDTA 1 mM, pH 8.0) to a working concentration of 25 ng mL-1 for PCR analysis.

Random Amplified Polymorphic DNA (RAPD) analysis

The RAPD analysis was performed following the methodology9 with slight variations. Primer used was synthesized by Qiagen Pvt Ltd. Sets of seven decamer primers were used for amplification of DNA. Condition were optimized for RAPD-PCR assay (Table 2a and 2b) using 50µl reaction (Bangalore GeNei^{TM}) containing 32.50 µl of autoclaved water, 1.5µl of dNTPs (dATP, dTTP, dCTP and dGTP), 7.50 µl 10X PCR buffer, 1.5µl of Taq DNA polymerase, 1µlof primer and 6µl DNA template (extracted DNA from 1ml of test sample dissolved in 50µl of TE buffer). The PCR amplification reaction was carried out in thermal cycler using the optimized programme (Table 3).

Restriction Endonuclease Digestion

Restriction Endonuclease Digestion of DNA was done with the help of GeNei ^{TM} kit. Two different reaction mixtures in 1.5 ml vials were prepared (Table 2c). Reaction 1 contained 20 µl test DNA, 25 µl 2X assay buffer and 3 µl EcoR I whereas Reaction 2 contained 20 µl test DNA, 25 µl 2X assay buffer and 3 µl Hind III . The vials were incubated at 37^0C for an hour. After an hour 5 µl of Gel Loading Buffer was added to each of vials to stop the digestion.

Table 2a: The master mixture for optimization of RAPD PCR (50 µl reaction volume)

NAME	VOLUME
Number of reactions	3
Reaction volume	50 µl
Total volume	150 µl
Autoclaved water	97.50 µl
10X PCR buffer with 15mm $MgCl_2$	22.50 µl
dNTP mixture	4.5 µl
Taq polymerase	4.5 µl
Primer	3 µl
Total volume	132 µl
Each reaction	44 µl master mix. + 6 µl isolated DNA

Table 2b: Samples preparation for RAPD PCR for isolated genomic DNA of two *Catharanthus roseus* cultivars

S. No.	Sample	Volume
1	Crw	44 µl of M.M + 6 µl of isolated DNA
2	Crp	44 µl of M.M + 6 µl of isolated DNA
3	CrBk	44 µl of M.M + 6 µl of D.D. water (sterile)
4	Total	176 µl +24 µl =200 µl

Now- Crw- *Catharanthus roseus* white, Crp- *Catharanthus roseus* pink,
CrBk- *Catharanthus roseus* blank, M.M- Master Mixture and D.D-Double Distilled.

Table 2c: Sample preparation for Restriction Endonuclease digestion of DNA

Reaction 1	Reaction 2
Crw genomic DNA - 20 µl	Crp genomic DNA - 20 µl
2X Assay buffer - 25 µl	2X Assay buffer - 25 µl
Eco RI -3 µl	*Hind* III -3 µl

Table 3: PCR programmed for RAPD (amplification of Isolated Genomic DNA)

Step	Temperature	Time	No. of cycle
1.Initial denaturation	94^0C	2min.	1
2. A- Denaturation	94^0C	30sec.	2
2.B-Annealing	35^0C	1 min.	
2.C-Extension	72^0C	2 min.	
3.A- Denaturation	94^0C	30 sec.	2
3.B- Annealing	35^0C	1 min.	
3.C- Extension	72^0C	2 min.	
4.A- Denaturation	94^0C	30 sec.	43
4.B- Annealing	35^0C	1 min.	
4.C- Extension	72^0C	2 min.	
5. Final extension	72^0C	5 min.	1
6.Hold	4^0C	As per convenience	1

Agarose Gel Electrophoresis

Genomic DNA (5 µl) with gel loading dye (3 µl) was separated in 0.8% agarose gel (Fig. 2). The amplicons were separated in 1.2% and 2 % agarose gel for RAPD (12 µl DNA + 3 µl dye) and Restriction Endonuclease Digestion (12 µl DNA + 3 µl dye) respectively. Electrophoresis was performed at a constant voltage at 60 V for 2 hours. The amplicons were visualized under UV light (Transilluminator Bangalore GeNei™) and photographed (Fig. 3 to 6). The amplicon size was determined by comparison with the ladder (Bangalore GeNei™ Ruler 100 bp ladder plus). The entire process was repeated twice to ensure reproducibility.

Fig. 2: Genomic DNA of two cultivars of *Catharanthus roseus* separated in 0.8% agarose

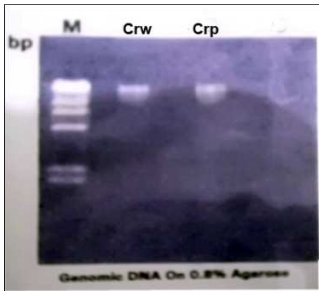

Fig. 3: RAPD PCR product (12 µl amplified DNA + 3 µl gel loading dye) using OP-04 primer, along with marker (M) at one side separated in 1.2% agarose

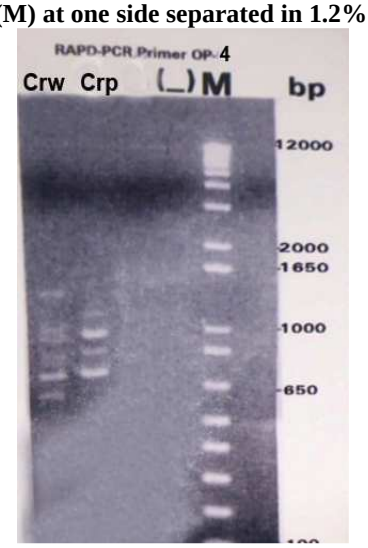

Fig. 4: RAPD PCR product (12 µl amplified DNA + 3 µl gel loading dye) using OP-07 primer along with marker (M) at one side separated in 1.2% agarose

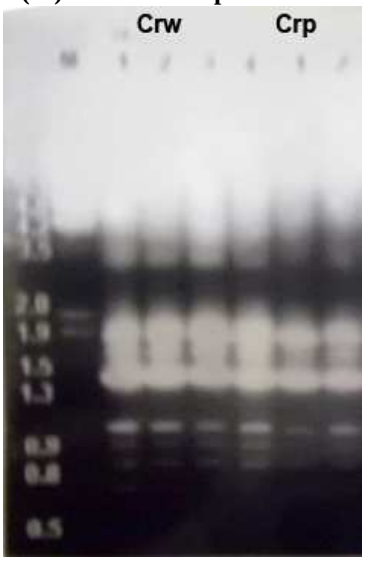

Fig. 5: Restriction Enzyme (*Eco*RI) digestion of genomic DNA runs over 2% agarose. Genomic DNA 5µl mixed with 3 µl gel loading dye and loaded in gel along with marker at one side

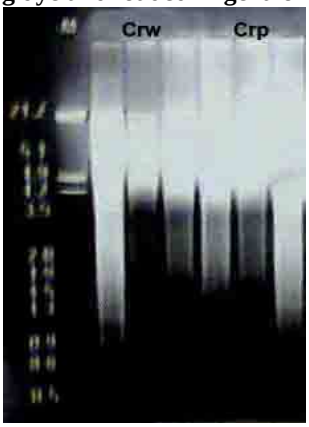

Fig. 6: Restriction Enzyme (*Hind*III) digestion of genomic DNA runs over 2% agarose. Genomic DNA 5µl mixed with 3µl gel loading dye and loaded in gel along with marker at one side

RESULTS AND DISCUSSION

In this investigation, seven random decamer oligonucleotide primers were used for two *Catharanthus roseus* cultivars. Out of these seven primers, the amplifications of only three primers (OP-04, OP-06 and OP-07) were satisfactory and reproducible. The reason for the non-amplifications of the other four primers could not be explained. Probably the sample DNA did not have any binding site for the primers. A similar nonamplification of decamer primers was reported [10,11,12,13] in different plant species. The amplification pattern is shown (Fig. 3, 4) and the details of the RAPD analyses (Table 4). All these 03 primers resulted in the amplification of 27 bands, indicating the presence of a high degree of genetic variation in the studied cultivars. All primers obtained a wide range of amplicons, ranging from 650 bp to 1750 bp. The largest amplicon (1750 bp) in this study was amplified by the primers OP-07 and the shortest (650bp) by OP-06. The highest number of amplicons (10) was observed for primer OP-07 and the lowest (8) for the primers OP-04. In this investigation, RAPD markers were successfully used to differentiate two cultivars of *Catharanthus roseus* from each other which are similar to observations [14, 15]. Our results also agree with findings[16] who used AFLP markers to study genetic diversity in *Caladium bicolar*.

Different banding pattern of Restriction Endonuclease Digested DNA showed (Fig.5, 6) that the DNA sequences of all the two cultivars of *Catharanthus roseus* are not same.

Thus, RAPD and Restriction Endonuclease are highly correlated with the combined markers suggesting that all markers are relevant to study the genetic diversity in *Catharanthus roseus.*

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