

## Optimization of RAPD-PCR Protocol to Screen *Jatropha Curcas* and *Gossypium Hirsutum* Grown in Metal Contaminated Soil

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### Abstract

The optimization of randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) protocol was performed to screen two plants (*Jatropha curcas* & *Gossypium hirsutum*) grown in metal contaminated soils. A CTAB method was employed for DNA extraction, but an overnight RNase treatment was carried out to eliminate RNA contaminants. The isolated DNA was used for RAPD-PCR amplification. The optimum condition for reliable amplification requires a higher concentration of MgCl<sub>2</sub> (3 mM), primer (2.5 μM), Taq DNA polymerase (1 unit) and 3 μl of template DNA (sample) and an annealing temperature of 55°C. Reproducible amplified products were observed in these conditions for both the plant species.

**Keywords:** *Jatropha curcas*; *Gossypium hirsutum*; DNA isolation; RAPD-PCR

### Introduction

In India and elsewhere in the world there are several hectares of wet land that was polluted by industrial activities such as mining, metallurgical processing, textile production, tannery etc., but these areas are in demand for cultivation for crop production. To reclaim these contaminated soils plants are grown (phytoremediation). While the plants used for phytoremediation on contaminated soil do rehabilitate the site, there may be a chance for changes in their DNA due to metal stress. This needs to be characterized, but there are few problems encountered during isolation and amplification of DNA from plants grown in metal contaminated sites. The metals could interfere with Taq DNA polymerase during amplification. Plants grown in this environment could contain inhibitory metabolites. Moreover, the contaminating RNA that precipitates along with genomic DNA could also cause suppression of PCR amplification [1], by improper priming of DNA templates. In recent years, molecular markers are increasingly being deployed due to study mutational changes. Among the different molecular markers, some are relatively cheaper and simple to use. One such marker is Random Amplified Polymorphic DNA (RAPD), which is a Polymerase Chain Reaction (PCR) based DNA marker. This assay is based on the amplification of genomic DNA with single primer of arbitrary nucleotide sequence [3]. RAPD is an inexpensive and rapid method, not requiring any information regarding the genome of plant, and has been widely used to ascertain the genetic diversity. It requires only small amount of genomic DNA and can produce high levels of polymorphism and may facilitate more effective diversity analysis in plants and it provides information that can help to define the distinctiveness of species and phylogenetic relationship. Various protocols are available for DNA extraction from plant species [4,5] and they have been further modified to provide suitable DNA for several kinds of analyses [6,7]. The ultimate aim of this study to isolate genomic DNA and identify an optimum RAPD-PCR condition to screen *Jatropha curcas* and *Gossypium hirsutum* collected from metal containing waste dumps of magnesite and bauxite mines.

### Materials and Methods

#### Plant material

The green house grown *J. curcas* and *G. hirsutum* was used for

phytoremediation on waste dumps of magnesite and bauxite mines. Two grams of young fresh leaves were harvested from these plants for genomic DNA isolation.

#### Reagent preparation

An extraction buffer consisting of 2% CTAB (w/v), 0.5 M Tris HCl pH 8, 0.5 M EDTA pH 8, 5 M NaCl, 0.2 g of poly vinyl pyrrolidone (PVP), 3 M Sodium acetate solution (pH 5.2), ribonuclease A (10 mg/ml), Chloroform: Isoamylalcohol (24:1), Phenol:Chloroform: Isoamylalcohol (25:24:1v/v/v), Ethanol (70%, 100%) and TE buffer (10 mM Tris HCl and 1mM, EDTA pH 8) were prepared.

#### DNA isolation protocol

The modified CTAB method of Padmalatha and Prasad [8] was adopted for the isolation of genomic DNA. Freshly harvested leaf sample (2 g) was ground in liquid nitrogen using a mortar and pestle along with 0.2 g of PVP [9]. The homogenized leaves were quickly transferred to 3 ml of freshly prepared prewarmed (65°C) extraction buffer and shaken vigorously by inversion to form slurry. The tubes were incubated at 65 to 68°C in a water bath for 60-80 min with intermittent shaking and swirling for every 30 min. Equal volume of Chloroform: Isoamylalcohol (24:1) was added and mixed properly by inversion for 30 min and centrifuged at 12,000 rpm for 15 min at room temperature to separate the phases. The supernatant was carefully removed and transferred to a new tube and an equal volume of icecold Isopropanol was added to form precipitation at -20°C for a minimum of 30 min. The samples were centrifuged at 12000 rpm for 15 min. The pellet was washed with 70% ethanol; air dried and resuspended

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in 3 ml of TE buffer and 5 µl of RNase was added and incubated at 37°C. The genomic DNA was extracted with equal volumes of phenol: chloroform: isoamylalcohol (25:24:1, v/v/v) at 8000 rpm for 15 min. The aqueous layer was transferred to a fresh 15 ml tube and extracted again with an equal volume of chloroform and isoamylalcohol (24:1). The genomic DNA precipitated using equal volumes of absolute alcohol and 1/10th volume of sodium acetate at -20°C for 30 min followed by centrifugation at 12,000 rpm for 15 min. The pellet was air dried and resuspended in TE buffer. All centrifugation steps were carried out at room temperature to obtain good quality of DNA without any impurities.

### Agarose Gel electrophoresis

The concentration and purity of isolated DNA was determined by running the samples on 0.8% agarose gel based on the intensities of band when compared with the Lambda DNA marker. The nucleic acid concentration was calculated following the method of Sambrook et al. [10].

### Primer used

Twenty primers from Operon Kit A (Operon - Medox -Bangalore, India) (OPA 01 to OPA 20) with 60-70% GC content were used (Table 1).

### Optimization of RAPD-PCR protocol

The genomic DNA of *J. curcas* and *G. hirsutum* were amplified using RAPD-PCR. The amplifications were carried out in a MyGene™ series Peltier thermal cycler Model MG 25+ (Long Gene Scientific instruments Co., Ltd). The various volume of samples (2,3,5,10,20,30,50,75 and 100µl of DNA templates), MgCl<sub>2</sub> (1, 2, 3, 4, and 5 mM), dNTPs (0.1, 0.2, 0.3 and 0.4 mM); primer (1, 1.5, 2, 2.5, 3, 3.5, 4.0, 4.5 and 5.0 µl), Taq DNA polymerase (0.1, 0.5 and 1 unit) and the total reaction volumes (14, 16, 18 and 20 µl) were tested. The thermo cycler was programmed for an initial denaturation step of various min (2, 3, 4 and 5) at 94°C, followed by various number of cycles: 25, 30, 35, 40, 45 and 50 cycles, and at various annealing temperature (45, 50, 55, 60, 65 and 70°C) for 1 min, extension was carried out at 72°C for 1 min and final extension at 72°C for 7 min and hold temperature of 4°C at the end. The amplified PCR products were electrophoresed on 1% (w/v) agarose gels in 1X TBE Buffer at 50 V for 3 h and then stained with ethidiumbromide (0.5 µg/ml). Gels with amplification fragments were visualized and photographed under UV light. The intact Lambda DNA (DNA) was used as a marker throughout study.

### Results and Discussion

The extraction of high quality and low polysaccharide genomic DNA from *J. curcas* and *G. hirsutum* taken from magnesite, bauxite and control soils was achieved. Peterson et al. [12] and Porebski et al. [13] stated that the presence of oxidizing agents (polyphenols), which are powerful compounds found in many plant species, can reduce the yield and purity by binding covalently with the extracted DNA making it useless for most research applications. Ziegenhagen and Scholz [7] reported some secondary metabolites (Tannins, terpenes and resins) are also difficult to separate from DNA and certain polysaccharides are known to inhibit RAPD-PCR. Many DNA isolation procedures also yield large amounts of RNA, especially 18S and 25S rRNA [2,14,15] from *Gossypium* species and *J. curcas*. Large amounts of RNA in the sample can chelate Mg<sup>2+</sup> and reduce the yield of amplified products (Table 1) (Figures 1,2,3). A prolonged

S.NO	Primers	Nucleotide sequence
1	OPA-04	5'AATCGGGCTG3'
2	OPA-13	5'CAGCACCCAC3'
3	OPA-18	5'AGGTGACCGT3'
4	OPA-07	5'GGTGACGCAG3'
5	OPA-01	5'TTCGAGCCAG3'
6	OPA-02	5'GTGAGGCGTC3'
7	OPA-03	5'GGGGGTCTTT3'
8	OPA-04	5'CCGCATCTAC3'
9	OPA-05	5'GATGACCGCC3'
10	OPA-06	5'GAACGGACTC3'
11	OPA-07	5'GTCCCGACGA3'
12	OPA-08	5'TGGACCGGTG3'
13	OPA-09	5'CTCACCGTCC3'
14	OPA-10	5'TGTCTGGGTG3'
15	OPA-05	5'TGTTCCACGG3'
16	OPA-07	5'CAGCGACAAG3'
17	OPA-16	5'TCTCCGCCCT3'
18	OPA-18	5'AATCGGGGAG3'
19	OPA-11	5'GTCCACTGTG3'
20	OPA-02	5'CACAGCTGCC3'

Table 1: Oligonucleotide Operon primers (A series).

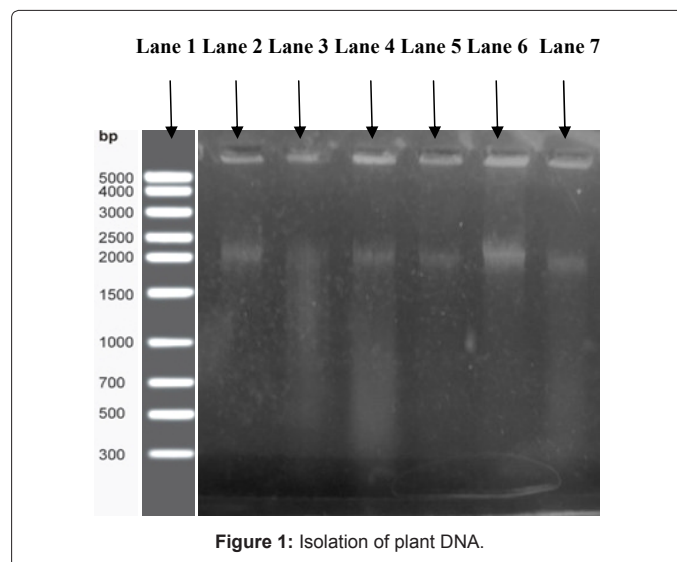


Figure 1: Isolation of plant DNA.

overnight RNase treatment degraded RNA into small ribonucleosides that do not contaminate the DNA preparation and yielded RNA-free pure DNA. Additional precipitation steps removed large amounts of precipitates by centrifugation and modified speed and time [11]. Almost all the tested parameters for RAPD-PCRs like the concentration of template DNA 3(µl), primer, magnesium chloride (3), Taq DNA polymerase (1), dNTPs (0.3) and temperature and time intervals during denaturation (3.0 min), annealing (55) and elongation (72°C for 1 min and final extension at 72 for 7 min) were also optimized, which also had an effect on amplification, banding patterns and

reproducibility. The optimized conditions for RAPD-PCR protocol are given in Table 2. The size of the amplified fragments ranged from 480-3300 bp. The conditions described in the present work consistently amplified DNA fragments of plant species belonging to different genera with various medicinal and aromatic properties. Liu et al. [16] conducted RAPD ‘fingerprinting’ technique and detected genotoxin-induced DNA damage of plants (barely) from heavy metal (Cd) contaminated soil and optimized the standard protocol. The present optimized protocol for DNA isolation and RAPD-PCR may serve as an efficient tool for further molecular studies. Padmalatha and Prasad [8] also reported that prolonged overnight RNase treatment to produce RNA-free pure DNA. Additional precipitation steps removed large amounts of precipitates (detergents, proteins and polysaccharides) by

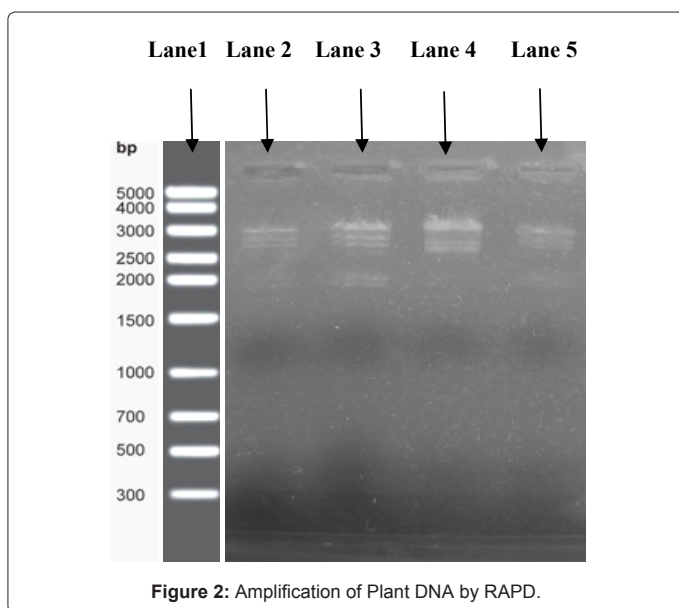


Figure 2: Amplification of Plant DNA by RAPD.

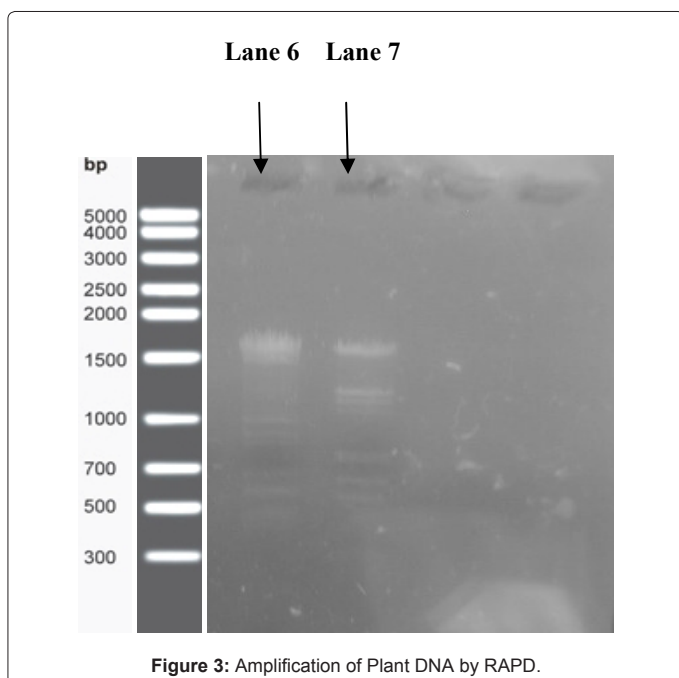


Figure 3: Amplification of Plant DNA by RAPD.

PCR reagents	Tested range	Optimum range	Results of tested ranges
<b>DNA concentration (µl)</b>	2,3,5,10,20,30,50,75 and 100	3	No amplified bands with lower concentration and presence of thinned band at higher concentration effected the repeatability
<b>MgCl<sub>2</sub> (mM) dNTPs (mM)</b>	1, 2, 3, 4, and 5 0.1, 0.2, 0.3 and 0.4	3 & 0.3	Excess/lower concentration increases the non specificity and yield of the product. Increased concentration reduces the free Mg <sup>2+</sup> , interfering with the enzyme.
<b>Primer concentration (µM)</b>	1, 1.5, 2, 2.5, 3, 3.5, 4.0, 4.5 and 5.0	2.5	Lower and higher concentrations lead to absence of amplification and primer dimer formation, respectively.
<b>Taq polymerase (Units)</b>	0.1, 0.5 and 1.0	1	Lower concentration did not show proper amplification. High concentration showed decreased specificity.
<b>Initial Denaturation time interval (min) at 94°C</b>	2, 3, 4 and 5	3.0	Higher/lower time intervals (from optimum) leads to reduction in amplification, loss of Taq polymerase activity and lack of reproducibility
<b>Annealing °C</b>	45, 50, 55, 60, 65 and 70	55	Higher/lower annealing temperatures (from optimum) results in difference in specificity
<b>Reaction volume (µl)</b>	14, 16, 18 and 20	16	Influences the cost of the PCR ingredients and not effective bands obtained
<b>Number of cycles</b>	25, 30, 35, 40, 45 and 50	30	The optimum cycles only shows effectiveness for amplification

Table 2: Optimization of the RAPD-PCR reaction for *J. curcas* and *G. hirsutum* from mine waste dumps.

centrifugation. DNA degradation and precipitations were avoided to some extent by carrying out all the steps at a perfect timing. We found that these modified steps are necessary to standardize and increase the quality and quantity of genomic DNA analyses of plants grown from metal contaminated soil.

## Conclusion

From our findings we concluded that the optimized protocol for plant genomic DNA isolation and DNA amplification process with Universal operon primers (A-serious) are effective for screening the plants grown from the metal contaminated sites.

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