DNA Extraction and Assessment of genetic variability through RAPD analysis for Molecular Studies in *Alstonia scholaris* (L.) R. Br.

E. Sreedevi[1]* and T. Pullaiah[2]

[1] Department of Botany, Govt. Degree College, Tadipatri, Anantapur (Dist.,), Andhra Pradesh, 515411, India

[2]Department of Botany, Sri Krishnadevaraya University, Anantapur, Andhra Pradesh, 515003, India.

Abstract

Plant Genetic analysis relies on high yields of pure DNA samples. Here a protocol was developed for the optimization of DNA isolation and PCR conditions for RAPD analysis of *Alstonia scholaris* (L). R. Br., a medicinal plant of Apocyanaceae for conservation concern containing high levels of alkaloids and secondary metabolites. The method involves a modified CTAB extraction employing liquid nitrogen for grinding and the powdered tissue was transferred to extraction buffer [100 mMTrisHCl (pH 8.0), 20 mM di-Sodium salt of EDTA, 2 % (w/v) CTAB, 1.4 M Sodium Chloride and 1 % (w/v) of Polyvinylpyrrolidone-40]. Later incubation in Chloroform: lsoamyalcohol mixture, RNase treatment and all steps carried out at room temperature. The quality of DNA was further tested by agarose gel electrophoresis. The yield of DNA ranged from 1049.1 and 877.2 ng (50 µl final volumes) from 3 gm of mature *Alstonia scholaris* leaf tissues and the purity (ratio) was between 1.7-1.8 indicating minimal levels of contaminating metabolites. The technique is ideal for isolation of DNA and the DNA isolated was used for randomly amplified polymorphic DNA (RAPD) analysis. RAPD protocol was optimized based on the use of higher concentration of $MgCl_2$ (2.5 µl), lower concentrations of primer (1.0 µl) and *Taq* polymerase (0.2 units), 2.0 µl of template DNA and an annealing temperature of 35°C, resulted optimal amplification. Reproducible amplifiable products were observed in all PCR reactions. Thus the results indicate that the optimized protocol for DNA isolation and PCR was suitable to plant species belonging to different genera with high levels of polysaccharides and polyphenolics and for processing large number of samples for genomic analysis, mapping and next generation sequencing.

Key Words: PCR, CTAB method, DNA Isolation, RAPD Analysis.

1. Introduction

Alstonia scholaris (L). R. Br., under the family Apocynaceae is one of the important medicinal plants and distributed throughout India. In Indian traditional system of medicine this plant is used to cure various diseases viz. asthma, cholera, diarrhea, malaria, pneumonia, tuberculosis (Jain, 1991), tumor, cancer (Pakrashi and Mukhopadhyay 2001), hepatitis, malaria, enlarged spleen, anthelmintic, antiepileptic, gonorrhea, asthma, lung cancer (Pullaiah, 2006; Mollik et al., 2010), etc. The enormous therapeutic importance and biological activities of *A. scholaris* is mainly due to great diversity of alkaloids (Pratap et al., 2013). Accurate and fast scientific identification of the plant(s) is the key to success for the herbal drug industry. The conventional approach is to engage an expert taxonomist, who uses a mix of traditional and modern techniques for precise plant identification (Mishra et al., 2016). Molecular genetics techniques using DNA polymorphism is increasingly used to characterize and identify a novel germplasm for uses in the crop breeding process (O Neill et al., 2003). Advances in molecular biology techniques have provided the basis for uncovering virtually unlimited numbers of DNA markers (Ahmad et al., 2010). Over the last decade, polymerase chain reaction (PCR) technology has become widespread research technique and has led to the development of several novel genetic assays based on selective amplification of DNA (Cao et al., 2010). Analysis of genetic variability and population structure of the endemic medicinal plants using molecular markers has been in practice (Ding et al., 2013). In contrast, molecular markers, based on DNA sequence polymorphisms are independent on environmental conditions and

show higher levels of polymorphism. DNA-based molecular markers are more efficient to analyze genotypic diversity than morphological markers and the knowledge gained from the analysis of plant genes is beneficial to all aspects of plant research, including crop improvement. Since 1990s, new methods and tools are continually being developed to facilitate rapid and accurate mapping, and analyzing of genes (Simko, 2016) among which random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), amplified fragment length polymorphism (AFLP), sequence-related amplified polymorphism (SRAP), and simple sequence repeat (SSR) analysis are well established and widely used (Shakeel et al., 2013 and Haridan et al., 2014). RAPD technique either alone or in combination with other techniques is widely used for the genetic characterization of different medicinal plants, and other organisms (Fu et al., 2013). Among polymerase chain reaction (PCR) based markers, random amplified polymorphic DNA (RAPD) markers are convenient and does not require prior knowledge of the DNA sequence to be amplified (Weder, 2002). The versatility of the PCR technique is that several kinds of sequencing primers can be explored for genome analysis depending on the purpose of study. The easy to access and low cost PCR-based markers include Random Amplified Polymorphic DNA (RAPD). RAPD markers have found a wide range of applications in gene mapping, population genetics, molecular evolutionary genetics and plant and animal breeding. This is mainly due to the speed, cost and efficiency of the RAPD technique to generate large numbers of markers in a short period of the compared with previous methods. The RAPD markers are easy to develop but lack of reproducibility makes it less reliable and obstacles to their further use in authentication of traits (Bhagyawat, 2016). The present work is thus the report on RAPD markers for assessing the underlying genetic variation in two morphological different genotypes of *Alstonia scholaris*.

2. Materials and Methods

Total genomic DNA was isolated by CTAB method as described by Rogers and Bendich (1994) and purified according to the following protocol. Extraction buffer, 10%CTAB, Precipitation buffer, High salt TE, 3M Sodium acetate, and 10 X TBE Buffer was prepared. Approximately 3 grams of leaf tissue was ground to a fine powder in liquid nitrogen. The powdered tissue was transferred to an Oakridge tube containing 5 ml of extraction buffer [100 mMTrisHCl (pH 8.0), 20 mM di-Sodium salt of EDTA, 2 % (w/v) CTAB, 1.4 M Sodium Chloride and 1 % (w/v) of Polyvinylpyrrolidone-40]. The contents of the tube were warmed at 65ºC for 15 minutes for sample 1 and 2 of two different genotypes. Two other samples 3 and 4 of the same genotypes were incubated at 65^0C for 45 minutes. The increase in incubation time could decrease turbidity due to latex and other polyphenolics and made easy further process of DNA isolation. Chloroform: Isoamyl alcohol (24:1) mixture was added to the solution and the contents were mixed vigorously. The tube was then centrifuged at 4290 x g for seven minutes. The aqueous phase was transferred to a fresh tube to which 1 ml of 10 % CTAB was added. The contents were mixed by vortex followed by addition of 5 ml of Chloroform: Isoamyl alcohol (24:1). The contents were shaken thoroughly and centrifuged at 4290 x g for seven minutes. The supernatant was collected and precipitation buffer [100 mMTrisHCl (pH 8.0), 20 mM di-Sodium salt of EDTA, 1 % (w/v) CTAB] was then added by an amount equal to three times of the volume of the supernatant. The contents were gently mixed and allowed to stand at room temperature for 30 minutes. The contents were centrifuged at 7627 x g for fifteen minutes. After discarding the supernatant, the pellet was allowed to dry and was resuspended in 500 µl of high salt TE [10 mMTrisHCl(pH 8.0), 1 mM di-Sodium salt of EDTA, 1 M Sodium Chloride]. The solution was transferred to 1.7 ml microfuge tube and centrifuged at 13,000 rpm in a microcentrifuge for two minutes to remove the insoluble debris. The supernatant was transferred to a fresh 1.7 ml microfuge tubes. To precipitate the DNA, two volumes of chilled absolute ethanol (95 %) was added and the tube was allowed to stand at -20^0C for one hour. Precipitated DNA was harvested by centrifugation at 13,000 rpm for fifteen minutes in a microcentrifuge. The DNA pellet was washed with 70 % chill ethanol/ kept on ice followed by another round of centrifugation at 13,000 rpm for five minutes. The supernatant was discarded and the DNA pellet was allowed to dry to remove traces of alcohol. The pellet was then dissolved in 500 µl autoclaved double distilled water.

Removal of RNA

5µl of RNAse added to the sample and kept in incubator for 30 minutes at 37^0C. The sample was spun at 13000 rpm and supernatant was collected. Phase partitioning by Phenol: Chloroform in an equimolar ratio was performed to free the DNA of contaminating proteins. For this we added equal volume of phenol: chloroform

and mixed by vortexing. This mixture was spun at 13000 rpm for 10 minutes and supernatant was collected. 500µl of Chloroform was added to the supernatant, vortexed to mix and centrifuged at 13000 rpm for 10 minutes. After removal of protein, (RNAse) the DNA was re-precipitated with absolute ethanol (95 %) kept on ice in the presence of 1/10 volume of Sodium Acetate (final concentration 3 M) and incubated at -20^{0}C for one hour. The precipitated DNA was harvested by centrifugation at 13,000 rpm for five minutes in a microcentrifuge. The supernatant was discarded and the pellet was washed with 70 % ethanol to remove any traces of salt. The DNA pellet was dried and dissolved in autoclaved double distilled water.

Quantification of DNA

The quality of DNA was further tested by agarose gel electrophoresis, on 0.8% agarose gel with 1X TBE buffer. Purified DNAs were quantified on 0.8% agarose gels were compared on gel with known concentrations of uncut lambda DNA. It was also quantified with Nano drop method.

Gel analysis

The integrity of DNA was judged through gel analysis in following steps-

- Casted agarose gel (0.8 %) 150 ml in 0.5 X TBE (Tris borate EDTA) buffer containing (0.5 g/ml) of ethidium bromide.
- 5 µl of DNA sample was loaded with a mixture of DNA loading dye.
- Loaded a known amount of uncut λ phage DNA as control in the adjacent well.
- The gel was subjected to electrophoresis at 50 V for 1.5 hours.
- DNA loaded in the wells was visualized under trans illuminator and the image was captured via GelDoc.
- Presence of a single compact band at the corresponding position of uncut phase DNA indicated high molecular weight of isolated DNA.

Nano Drop method

The quantization of DNA was carried out by taking reading at 260 nm and 280 nm wavelengths by using a spectrophotometer (Thermoscientific Nano Drop ND-2000) in following steps-

- 2 µl of double distilled water was loaded in the lower pedestal to initialize the spectrophotometer
- The wavelength was fixed at 260 and 280 nm.
- The 2 µl of sterile Double distilled water was loaded for blank measurement.
- The quantities of unknown samples were recorded as ng/µl of genomic DNA.
- Appropriate dilutions of DNA (25ng/100µl) from the above stock were made in autoclaved double distilled water and stored in -20^{0}C refrigerator for future use.

RAPD analysis

For RAPD analysis a set of 6 random decamer oligonucleotide primers (series OPA, OPB) purchased from Operon technologies Inc. (Alameda, USA) was used for amplification of RAPD fragments. The primer sequence information was obtained from kit. The PCR reactions were performed in a 25 µl reaction mixture containing: Distilled water 14.3 µl, Taq Buffer 2.5 µl (10X Assay Buffer, $MgCl_2$ 2.5 µl, dNTPs 2.5 µl (200 µM each of dNTPs (Genedirex), Taq pol. 0.2 U, Primer 1.0 µl (OPA Set # 4, OPB Set # 2), Template 2.0 µl. The PCR reactions were carried out in DNA thermal cycler (Model-Applied Biosystems 2720, Foster city, USA) using a single primer in each reaction following the cycling parameter : Step 1: Initial denaturation (94ºC) for 5 Minutes, Step 2: (Cycle 1 – 45), Denaturation (94ºC) 1 Minute, Primer annealing (35ºC) 1 Minute, Primer Extension (72ºC) 2 Minutes, Step 3: Final extension (72ºC) 10 Minute. Following the amplification, the PCR products were loaded on 1.2 % Agarose gel (Himedia, molecular grade) which was prepared in 1X TBE buffer containing 2 µg/ml of Ethidium bromide. The amplified products were subjected to agarose gel electrophoresis for 3 - 3.5 h at 90 V. After separation the gel was viewed under UV trans-illuminator and the image was captured via GelDoc. Data scoring and Analysis carried by the presence of a particular band was scored as 1 and absence as 0.

3. Results

This method modified from basic CTAB DNA extraction protocol yielded high quality of DNA for leaf tissues of both the genotypes. The total DNA yield was 1049.1 and 877.2 ng (50 µl final volumes) from 3 gm of mature *Alstonia scholaris* leaf tissues (Table 1). Quality and quantity of DNA was determined based on the absorbance at *A260* and *A280* ratio and 0.8% agarose gel electrophoresis. The absorbance of isolated DNA was

1.8 at *A260/A280* indicating that it did not have any RNA or protein contamination (Varma et al., 2007). Initial incubation at 65^0C for 45 minutes improved the DNA yield and quality by preventing the sample from becoming viscous in nature during further steps in DNA isolation when compared to incubation at 65^0C for 15 minutes. Agarose electrophoresis results also suggest that incubation at 65^0C for 45 minutes improved DNA extraction method produces high quality genomic DNA without shearing and RNA contamination (Fig. 1).

Table 1. Quantification of DNA of *Alstonia scholaris* by using a NANODROP spectrophotometer

Sample ID	ng/µl	A260/280	Remarks
1 (at 65^0C for 15 minutes)	2217.1	1.86	Light pink in colour
2 (at 65^0C for 15 minutes)	2695.3	1.69	Turbid
3 (at 65^0C for 45 minutes)	1049.1	1.77	Crystal clear
4 (at 65^0C for 45 minutes)	877.2	1.81	Crystal clear

Figure 1. Agarose electrophoresis of total genomic DNA extracted from mature *Alstonia scholaris* leaf tissue resolved on 0.8% agarose [Lanes1-2: DNA from two different genotype plants incubation at 15^0C, 3-4: DNA from two different genotype plants incubation at 65^0C, 5: uncut λ DNA (100ng)]

The suitability of this extracted genomic DNA as a template in PCR amplification reactions was analysed with RAPD analysis with primers OPA and OPB series to compare band standards and to test the efficiency of the methodologies for future studies (Fig.2). The amplified products of these six primers were run on 1.2% agarose in 1X TBE buffer.

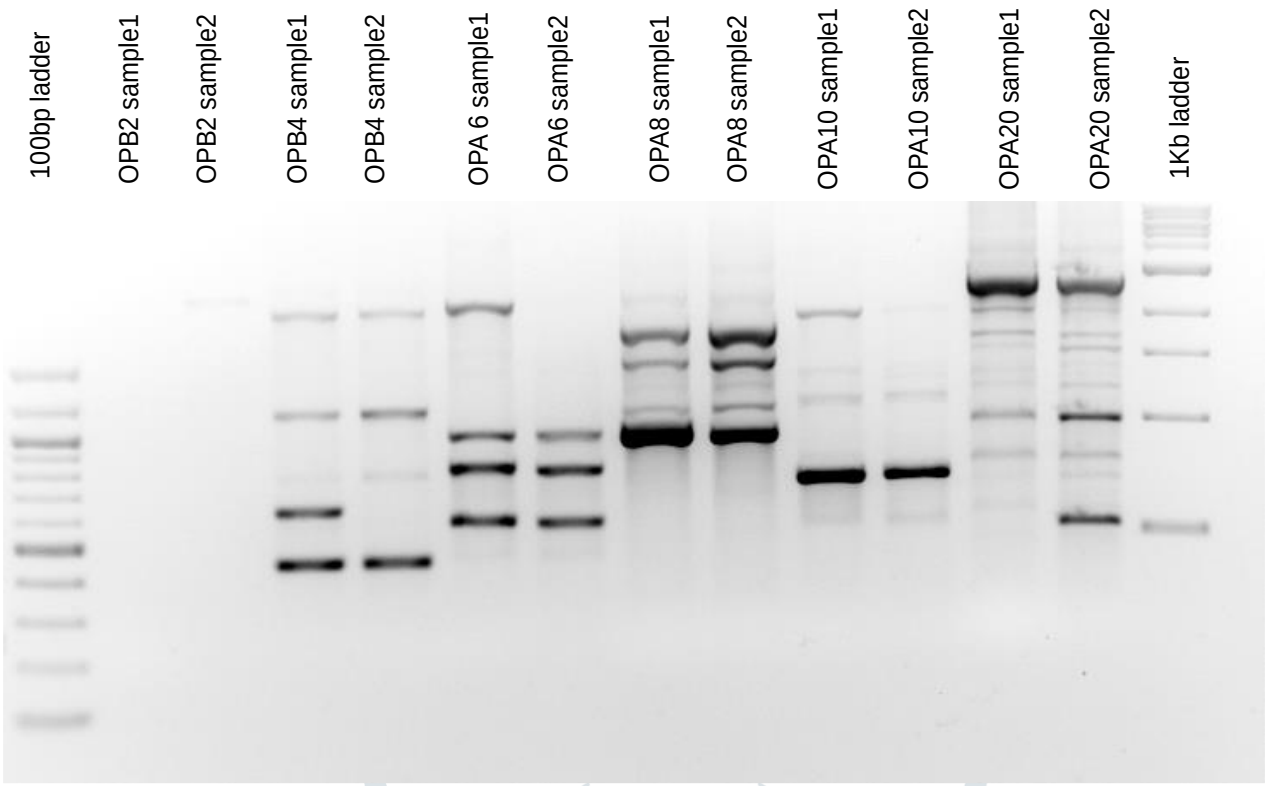

Figure 2. RAPD banding pattern of *Alstonia scholaris* with arbitrary primers

After screening it was observed that the molecular weights of the amplified products were in the range of 450bp to 2800bp. 52 bands were found to be polymorphic and showed that RAPDs are an abundant source of polymorphic markers in Apocyanaceae. The banding pattern obtained showed high genetic variation among individuals of the same species. Although the bands varied in size, the results from each RAPD product were assumed to represent a single locus and data was scored as the presence (1) or absence (0) of a DNA band. Only those fragments that amplified consistently were considered for analysis (Table 2).

Table 2. DNA Band polymorphism in two genotypes of *Alstonia scholaris* with different primers

Primers	Size (bp)	Genotype	
		1	2
OPB2	2800	0	1
OPB4	2700	1	1
	1100	1	1
	720	1	1
	640	1	0
	450	1	1
OPA6	2700	1	0
	900	1	1
	720	1	1
	550	1	1
OPA8	1900	1	1
	1500	1	1
	1300	1	1
	1100	1	1
	910	1	1
OPA10	2000	1	1
	1700	1	1
	1300	1	1
	650	1	1
OPA20	2900	1	1

	2000	1	1
	1700	1	1
	1500	1	1
	1400	1	1
	1300	1	1
	1000	1	1
	800	1	1
	550	0	1

High level of polymorphism was generated by OPA6 (25.00%), while minimum number of polymorphic bands were produced by OPA20 (11.11%) among the samples tested (Table. 3).

Table3. Details of primers, bands and polymorphisms in *Alstonia scholaris* as revealed by 6 primers used in RAPD-PCR

Primers	Sequence	Total No. of bands (a)	Total No. of polymorphic Bands(b)	Amplification size (bp)	Polymorphism (b/a*100)
OPB2	5'TGATCCCTGG3'	1	0	_	0%
OPB4	5'GGACTGGAGT3'	5	1	640	20.00%
OPA6	5'GGTCCCTGAC3'	4	1	2700	25.00%
OPA8	5'GTGATCGCAG3'	5	0	_	0%
OPA10	5'GTGATCGCAG3'	4	0	_	0%
OPA20	5'GTTGCGATCC3'	9	1	550	11.11%

4. Discussion

In the present study the method developed for isolation of DNA can be performed in any moderate laboratory for many applications such as *in vitro* regenerated culture of plant cells, tissues or organs. Alstonia scholaris has large amount of secondary metabolites and milky sap having several medicinal properties. Molecular studies dealing with medicinal and aromatic plants are rare in comparison with other cultivated plants due to the presence of medicinal compounds, which inhibit DNA amplification in PCR reaction (Mizukami and Okabe, 1999). Protocol by CTAB method as described by Rogers and Bendich (1994) gave good results and developed a procedure for high quality of DNA for PCR and amplification purposes and this is different to earlier reports where Richards (1997) protocol followed (Mahmood et al., 2011). The quantity and quality of DNA was confirmed by NANODROP Spectrophotometer and agarose gel electrophoresis. The increase in incubation time from 15 to 45 minutes at 65^0C could decrease turbidity due to latex and other polyphenolics and effective in producing samples that did not have any RNA or protein contamination for further process of DNA isolation. But the absorbance of isolated DNA was not varied significantly at *A260/A280* in both procedures.

In the present study, for RAPD-PCR the six primers namely OPB2, 4, OPA6, 8,10 and 20 were used for amplification. Te banding pattern score was based on the presence or absence of clear, visible and reproducible bands. Te results were analyzed based on the principle that a band is considered to be 'polymorphic' if it is present in some individuals and absent in others, and 'monomorphic' if present in all the individuals. In this study, all the primers produced a total of 1, 9, 7, 10, 8 and 17 bands respectively. The primer OPA6 showed high degree of polymorphism compared to OPA20 and OPB4 suggesting that it could be used as suitable molecular markers for diversity analysis. The technique employed in this study has the advantage of being inexpensive to perform, and does not require a previous knowledge of the genome. These genetic diversity analysis results were different to the earlier reports which is due to variation in marker selection i.e. the Primers OPC 2, 4, 5, 6, 8 and 9 were used by Mahmood et al. (2011).

RAPD fingerprinting was used for the detection of variety (Nandkumar et al., 2017) and clonal variation of plant species *in vitro* and *in vivo* (Wang et al., 2011; Arif et al., 2010). Thus, the use of RAPD markers for

taxonomic and systematic analyses of plants (Bartish et al., 2000) as well as in the study of genetic relationships has increased (Ranade et al., 2001)

Therefore, RAPD technique can be performed in a moderate laboratory for many applications mentioned above. Many useful applications of RAPDs have been reported in establishing the genetic stability of cultivated as well as *in vitro* regenerated culture of plant cells, tissues or organs in many other plant species viz., plants such as *Andrographis paniculata* (Ghosh et al., 2014), *Garcinia* (Bheemaiah et al., 2018), *Cyclanthus bipartitus* (Kasim et al., 2018).

5. Conclusion

A simple and efficient high quality DNA extraction method was optimized by CTAB method for the tree species *Alstonia scholaris.* The principal advantage of this protocol are its short duration with reduced completion time yielding high quality DNA, and is comparatively cheaper than most of the costly commercial kits. Also, this method is highly suitable for extracting high quality DNA from plants with high levels of polysaccharides and polyphenolics and for processing large number of samples for genomic analysis, mapping and next generation sequencing. RAPD markers were used to assess the underlying genetic variation in two morphological different plant types of *Alstonia scholaris*. The results showed that RAPD markers could be used with high rates of success for studying genetic variation in *Alstonia* species. This method will be used in my further research on variability. It helps in detailed study desirable to understand all the aspects related to variations.

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