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Extraction of PCR-usable DNA from trees adapted to arid environment

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Abstract

Genetic conservation programs in arid natural repertoire rely on molecular methods for diversity assessments. Molecular characterization involves the use of high molecular weight genomic DNA as starting material. Obtaining intact genomic DNA of sufficiently high quality, readily amplifiable using PCR is a primary goal of all molecular genetic studies. The aim of our study was to devise a method for the isolation of the genomic DNA from arid tree species viz. *Prosopis cineraria*, *Calligonum polygonoides* and *Acacia nilotica* (which are rich in polysaccharides, polyphenols and secondary metabolites), hold promises for the restoration of the region. The quality checking of the isolated DNA samples was done through the 10-mer oligonucleotide primers. The present method involves a modification of the available CTAB method employing higher concentration (6%) of polyvinylpyrrolidone (PVP), an extrinsic factor for reducing the metabolite precipitation, higher concentration of CTAB (3%) and an increased period (45 minutes) of incubation with chloroform: isoamylalcohol followed by the RNAse treatment for 90 minutes at 37°C and subsequent pelleting in TE buffer. The yield of DNA ranged from 1240-1500ng/µl and the absorbance lies between 1.7-1.9, indicating minimal levels of contaminating metabolites. The protocol has been tested with three tree species of the arid region which are extremely drought resistant. The DNA isolated was successfully amplified by all the random 10-mer oligonucleotide primers tested with high reproducibility. Optimum annealing temperature was 35°C at a concentration of $MgCl_2$ (1.5mM), lower concentrations of primer (0.2µM) and *Taq* polymerase (1U). The present method is simple, efficient and economically yielding good quality intact genomic DNA suitable for large genetic screening programs.

Keywords: *Prosopis cineraria; Calligonum polygonoides; Acacia nilotica;* polyphenols; polysaccharides; DNA extraction; PCR.

Abbreviations: CTAB_Cetyltrimethylammonium bromide; EDTA_ethylenediaminetetraacetic acid; PVP_ polyvinylpyrrolidone; RAPD_Random amplified polymorphic DNA; ISSR_Inter simple sequence repeats; AFLP_Amplified fragment length polymorphism; RFLP_Restriction fragment length polymorphism; SSR_simple sequence repeats; SNP_Single nucleotide polymorphism; IRAP_Inter-Retrotransposon Amplified Polymorphism; REMAP_Retrotransposon-Microsatellite Amplified Polymorphism.

Introduction

The potential of biotechnology to substantially benefit arid areas mainly deflation zones which are at risk due to the consequences of aeolian erosion and fluvial erosion can be demonstrated clearly through its ways of characterizing and conserving the woody tree species (*Prosopis cineraria*, *Calligonum polygonoides* and *Acacia nilotica*) that have reached a distraught condition due to the ruthless and

deliberated activities, leading to the biodiversity loss as well as extending the list of the threatened plants in the IUCN. *Prosopis cineraria.* (Linn.) Druce commonly known as Khejra, Khejari or Sangria, is one of the most common trees of the desert area belonging to Mimosaceae. *P.cineraria* show great genetic variability in mean biomass productivity per tree, in quality and quantity of fodder and pod production, making it an out crosser (Simpson, 1977). *Calligonum polygonoides* (Linn.)*,* one of the most dominant biomass producer of the sandy areas of the desert, belonging to Polygonaceae, locally known as Phog or Phogra, has remarkable adaptability to the adverse environmental conditions. The plant has been categorized as an endangered Plant in Red Data Book of India. *Acacia nilotica (*Linn.) Wild .ex Del., commonly called as "Babool" or "Kikar". Desi – Babul, Egyptian Thor, is a moderate to large size multipurpose nitrogen fixing tree distributed to arid and semi-arid regions of the country having fuel, metabolites of medicinal value and gums as its powerful implements in various applications. These plants have been known to contain high levels and different types of secondary metabolites, which are often responsible for their therapeutic and pharmacological activities. The elicitation of the particular metabolite through the use of the elicitor's during the growth stage of the plant helps in making plant based factories for pharmaceutical production, urging the need for the plant conservation (Pirttilä et al., 2001). PCR, most exploited analytical method in plant genetics for indexing the status of the genetic diversity and identification of quantitative trait loci (QTL). A number of molecular methods including the dominant (RAPD, ISSR and AFLP) as well as co-dominant marker (RFLP,SSR,SNP,IRAP,REMAP) approach have their roles well established in the analysis and prediction of genetic diversity and establishing the lines of evolutionary lineage. The application of the molecular methods has been further exploitated for the certification of the genetic fidelity of the clonally propagated plants and also for genome stability studies (Gesteira A. S. et al., 2002, Yerramsetty Praveen Nagh et al., 2008). The classical approach to the analysis of genetic variations across genomes- (RFLP) followed by a novel and enhanced approach (PCR) require a sufficient amount and quality of nucleic acids for the applicability of these assays. However, high amounts of polysaccharides, polyphenols and various secondary metabolites such as alkaloids, flavonoids and tannins especially in tree species usually interfere with DNA isolation (Mishra et al., 2008). Cell lysis process followed by the polyphenols oxidation and co-precipitation causes the browning of the DNA (Varma et al., 2007). Viscous DNA samples have been obtained as a result of the co-precipitation of the gelling polysaccharide making the samples viscous and hampers the proper loading of the samples on to the gel for electrophoresis (Diadema et al., 2003; Varma et al., 2007). Several protocols for genomic DNA isolation in plants having high polyphenolic and polysaccharide precipitation have been published but with no universality (Varma et al., 2007). Because most of the available procedures are based on the use of commercial kits which make routine usage extraction economically difficult for large scale genomic applications. We report an improved method of DNA extraction from plants with high levels of polyphenols & polysaccharides. A good isolation protocol should be simple, rapid and efficient enough, yielding appreciable levels of high quality DNA suitable for molecular studies. Because most of the available procedures are based on the use of commercial kits which make routine usage extraction economically difficult for large scale genomic applications. We report an improved method of DNA extraction from plants with high levels of polyphenols & polysaccharides for these selected tree species.

Mateials and methods

Plant materials

Prosopis cineraria, Calligonum polygonoides and *Acacia nilotica* foliate only for 2 to 3 months during the months (March to May) and rainy season, in addition the terrain and climatic conditions of the sampling area is very inhospitable. Individual plants were chosen randomly to provide a representative sample of the occupied territory from the Candidate plus Tree (CPT) sites. Fresh and dried samples of Candidate plus Trees (CPT) of *Prosopis cineraria, Calligonum polygonoides* and *Acacia nilotica* were collected from Nagaur, Jodhpur and Bikaner districts in Rajasthan, India. The position of the plant was noted down, and the plants were tagged. The leaves were brought to the laboratory in an icebox and were fixed in the pre-chilled mortar pestle using liquid nitrogen stored in cryocan BA-7.

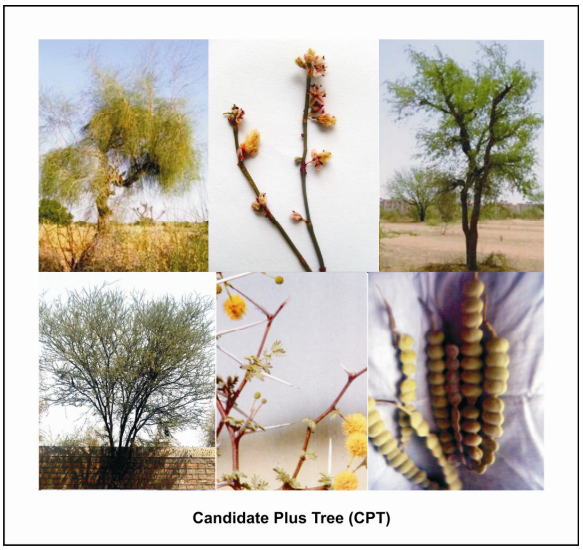

Fig a: Showing candidate plus trees (CPT) used for molecular study.

Reagents and Chemicals

The chemicals and reagents used in the isolation of DNA were: CTAB extraction buffer [3% (w/v) CTAB; 20 mM EDTA, pH 8.0; 100 mM Tris–HCl, pH 8.0; 4 M NaCl, TE buffer [10 mM Tris–HCl, pH 8.0; 1.0 mM EDTA, pH 8.0]; Chloroform: Isoamyl alcohol (24:1, v/v); Tris-Phenol: Chloroform: Isoamyl alcohol (25:24:1,v/v); Iso-Propanol; Absolute Ethanol; 80% Ethanol; PVP; β-mercaptoethanol (β-ME).

DNA extraction procedure

CTAB method (Doyle and Doyle, 1990) with slight modification was followed for DNA extraction. Plant materials were frozen in liquid nitrogen (−196°C) in a sterile mortar and ground to a fine powder. 1 g of frozen leaf tissue powder was transferred to a 1000 µl of pre-heated (65°C) CTAB DNA extraction buffer (100 mM Tris HCl, 4 M NaCl, 20 mM EDTA,3% CTAB,6% PVP and 0.2% β-mercaptoethanol) in a 2 ml sterile Eppendorf tube. The samples were given 10-15 minutes gentle inversions. The homogenates were incubated in a warm water bath maintained at 65°C for 30 minutes with intermittent swirling. After incubation, the homogenates were brought to room temperature and Tris-Phenol: Chloroform: Isoamyl alcohol (25:24:1, v/v) was added in equal volume to the homogenates and mixed gently by inversion, followed by centrifugation at 10,000 rpm (7500 g) for 15 min. The aqueous phase was pipetted into a sterile new eppendorf tube vial. The aqueous phase was re-extracted with chloroform: iso-amyl alcohol (24:1) firstly by incubating them for 45 min and then several inversions were made and then centrifuged at 10,000 rpm (7500 g) in a refrigerated centrifuge at 4°C. The aqueous phase was separated and the contaminating RNA was removed by incubating the samples with 5 µl of RNase (20mg/ml) for 90 minutes at 37°C. The DNA was precipitated by adding one tenth volume of the sodium acetate (pH 5.2) and three volume of the chilled absolute ethanol and then were inverted several times to bring effective precipitation. The samples were given incubation at -20°C for overnight and then the samples were centrifuged at 10,000 rpm (7500 g) at 4°C. DNA pellet was recovered by decanting the supernatant followed by washing twice with 70 % ethanol. The pellet so obtained has been dried under vacuum using Speed Vac (Thermosavant) at 37°C for 10 minutes. The pellet was re-suspended in TE buffer and stored at −20°C until further use.

DNA quantification

The quantification of genomic DNA was achieved using a spectrophotometer (UV–Visible Elico spectrophotometer). The yield was determined by measuring the absorbance at A_{260} and A_{280}. The level of DNA purity was determined by the A_{260}/A_{280} absorbance ratio. The ratio ranges between 1.7-1.9. DNA purity was further tested by electrophoresing the extracted genomic DNA (gDNA) on 0.8% agarose gel at 100 V for 120 min in 1×TAE (Tris Base, Glacial Acetic Acid, 0.5 M EDTA) gel buffer in a Submerged Agarose Gel Electrophoresis (AE-6125 / 6133 ATTO, Japan). The gel was stained with 0.25 µg/ml ethidium bromide for the visualization of gDNA. The gels were visualized and photographed using the Gel Doc fitted with 8 bit CCD camera and UV light. (Syngene Gel Doc, Syngene, Synoptics Ltd.).

SPAR-Single primer amplification reaction

DNA amplification reactions were assembled in 25 µl volumes containing 10 ng of template DNA, 1× PCR buffer (100mM Tris (pH 9.0), 500mM KCl, 15mM $MgCl_2$ and 1% Triton-X-100), 100 mM each of dATP

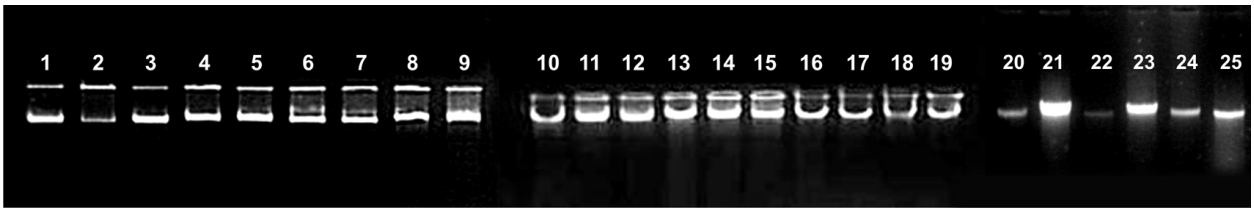

Fig 1. Genomic DNA extracted by modified CTAB method from 9 accessions of *Prosopis cineraria* (lane 1-9), 10 accessions of *Calligonum polygonoides* (lane 10-19) and 6 accessions of *Acacia nilotica* (lane 20-25)

dCTP, dGTP and dTTP (Bangalore Genei, India) and 1U *Taq* DNA polymerase (Bangalore Genei, India) The primer were added to a final concentration of 0.2 µM (Primers P1-5'-CAGCACCCAC-3'; P2-5'-CCCGATTGGG-3'; P3-CCCGAAGCGA-3'; P4-5'-CCGTCGGTAG-3'; P5-5'-GGTGCGGGAA-3'; P6-5'-GTTTCGCTCC-3'; P7-5'-GTAGACCCGT-3') (Williams et al., 1990). The samples were amplified in a thermal cycler (Eppendorf 5331) through 40 cycles of initial denaturation for 2 min at 94^{O}C; denaturation for 20 sec at 94^{O}C; annealing for 1 min at 35^{O}C; elongation for 2 min at 72^{O}C followed by final extension for 10 min at 72^{O}C. The amplification products for all plant species were resolved on 1.2% low EEO agarose gel. Reproducibility of these selected primers was evaluated using three independent PCR reactions. Negative control PCR reactions having all required PCR components except template DNA were also included.

Results and Discussion

Genomic DNA amplification, Library construction and Cloning of the genes for indexing the genetic diversity of arid regions requires the isolation of high quality intact DNA free from the endogenous secretion of polyphenols and viscosity. In a comparative analysis of different DNA extraction protocols from plant tissues, it has been stated that "the problem of DNA extraction is still an important issue in the field of plant molecular biology" and that "a chemical tissue disruption method as used in mammalian cells might be the method of choice"(Csaikl UM et al., 1999). Arid trees have high levels of polysaccharide, phenolics and other organic constituents and metabolites with medicinal properties and used in food, pharmaceutical and cosmetics (Khanuja et al., 1999). However these molecules pose great problem when DNA-based studies are performed which require high quality purified DNA. The problem of polyphenols intensifies when older trees are sampled. Many CTAB and PVP based protocols have been tried to isolate the high quality DNA from the tree species (Kim CS et al., 1999). Standard methods of gDNA isolation (Dellatropa, 1983; Saghai-Maroof et al., 1984) yield standard amount of gDNA but with high level of the protein and polyphenols contamination. The A260/280 absorbance ratio for the standard method (Dellatropa, 1983; Saghai-Maroof et al., 1984) lies between 1.6-1.8 indicating the level of contaminating molecules. These contaminating molecules precipitate along with DNA thus considerably affecting DNA yield quantitatively and qualitatively (Fang et al., 1992; Pandey et al., 1996).

A decrease in the number of amplicons in the amplified gDNA suggests presence of polysaccharide and polyphenols as well as RNA, which inhibits Taq polymerase (Scott and Playford, 1996). Polyphenol metabolite precipitation (Katterman and Shattuck, 1983) during the isolation procedure due to polyphenolic oxidation acts as a competitive inhibitor for the activity of the restriction enzyme. Beside these facts we established a protocol for the isolation of genomic DNA from the trees *Prosopis cineraria, Calligonum polygonoides* and *Acacia nilotica.* We tried several protocols but the DNA yield remained unsatisfactory in terms of quality and amplifiability. In our method higher period of incubation at 65°C result in lower yield of gDNA. PVP forms the complexes during the isolation of the aqueous phase after incubation with CIA (Chloroform: Isoamylalcohol) (Maliyakal EJ., 1992). Higher concentration of PVP (6%) has been found effective in reducing the polyphenols and pigmentation of DNA samples. Longer period of incubation (45 minutes) with Chloroform: Isoamylalcohol results in the clear white pellets of DNA. The modified protocol was tested with extensive germplasm

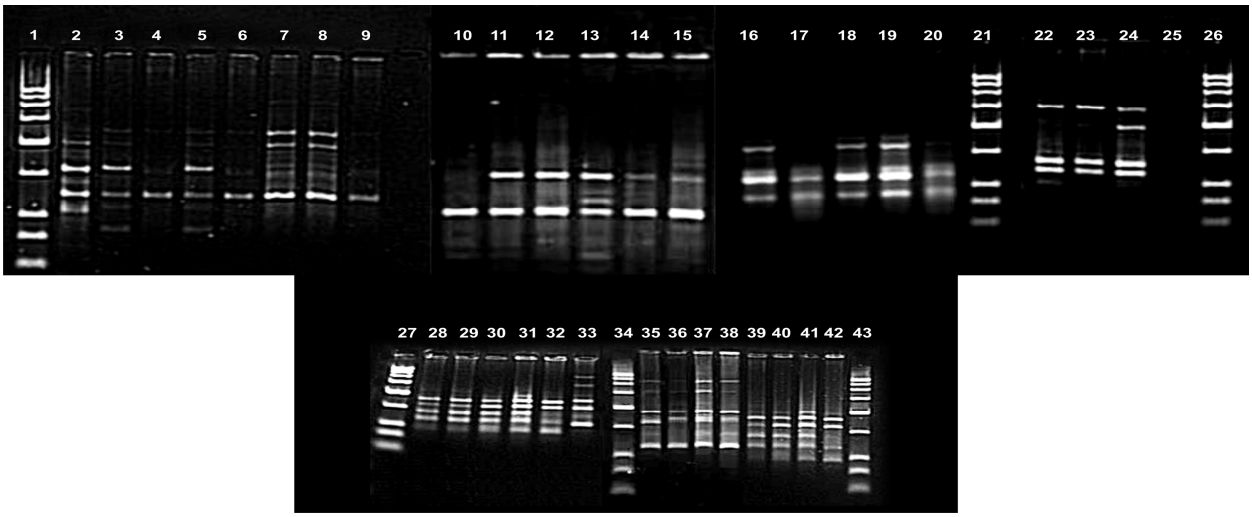

Fig 2. SPAR profile of different accessions of three arid tree species *C. polygonoides* (lane 2-9 &22-24), *P. cineraria* (lane 10-20) and *Acacia nilotica* (lane 28-33,35-42). Lane 2-10, Genomic DNA of eight accessions of *C. polygonoides*, amplified with primer 1. Lane 10-15, six accessions of *P. cineraria*, amplified with primer 2. Lane 16-20, five accessions of *P. cineraria*, amplified with primer 3. Lane 22-24, three accessions of *C. polygonoides*, amplified with primer 4. Lane 28-33, Genomic DNA of six accessions of *Acacia nilotica,* amplified with primer 5. Lane 35-38, Genomic DNA of four accessions of *Acacia nilotica,* amplified with primer 6. Lane 39-42, Genomic DNA of four accessions of *Acacia nilotica*, amplified with primer 7.Lane 1, 21 and 26, 27, 34, 43- molecular size marker in kb.

collections for reproducibility and compared with previously established protocols like CTAB (Doyle and Doyle, 1990) and combination of CTAB and SDS (Keb-LLanes et al., 2002). In comparison to other protocols, this method efficiently removed polysaccharides and the DNA pellet, obtained readily dissolved in TE, devoid of inhibitory polysaccharides. The A260/280 ratio ranged between 1.7 and 1.9. Our improved method yields high DNA content (Fig 1), which is polyphenols free and show no signs of degradation. The quantity of the DNA isolated is 1240-1500ng/µl of plant material. To test the purity and amenability of the isolated DNA, successful amplification was done with several random10-mer oligonucleotide primers (Fig 2) resulting in monomorphic bands as well as the polymorphic patterns at intra-zonal levels. The published protocol of Doyle and Doyle (Doyle and Doyle, 1990) is successfully optimized by increasing the concentration of CTAB, PVP and high NaCl to the gDNA extraction buffer. We are carrying out further studies to catalogue the genetic diversity in the arid region species using both ITS- PCR -RFLP and EST-SSR based approaches.

Fig b. Geographical localization of studied state and surveyed regions.

Conservation Implications and Conclusions

Overexploitation and uncontrolled utilization of *Prosopis cineraria, Calligonum polygonoides* and *Acacia nilotica* plants for commercial reasons are continuously eroding genetic potential of the species, need to be marked. In conclusion, the current method is simple and reliable for the isolation of genomic DNA from fresh and dry accessions of *Prosopis cineraria, Calligonum polygonoides* and *Acacia nilotica*. The protocol is simple, quick and is easily amenable to other hard-hitting tree species playing major roles in carbon-mitigation and bioenergy resources despite the complexity of their genomes. Our procedure is not only very simple, but is also sample, time and cost effective. Since this method does not involve expensive commercial DNA extraction kits, leading to its quick applicability in low budget laboratories.

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