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A SIMPLE AND EFFICIENT METHOD FOR HIGH QUALITY GENOMIC DNA ISOLATION FROM *Cannabis sativa* CONTAINING HIGH AMOUNT OF POLYPHENOLS

Running Title: DNA extraction and RAPD-PCR optimization of *Cannabis sativa*

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ABSTRACT

Isolation of high quality DNA from plants has been a challenging effort involving versatile protocols and methodology since long. Present methods of DNA isolation from plants have their own limitations such as highly sophisticated procedures, contamination of DNA with secondary metabolites and a lesser amount of isolated DNA. Through this paper, a novel method of DNA isolation has been presented that is quick, inexpensive and consistent protocol for extraction of DNA as compared to contemporary counterparts. DNA obtained; from leaf material containing large quantities of polyphenols, tannins and polysaccharides was intact and of quality (260/280 = 1.8 ± 0.02) routinely yielding 89.5 µg/ml. DNA extracted using this process is reproducible, free from contamination and is found to be suitable for PCR analysis particularly where large number of samples are to be analyzed. Isolated DNA has been characterized though various techniques and is found to be suitable for plant genetic studies, with significant amounts of compounds present in plant tissues that may interfere with subsequent DNA manipulation. RAPD-PCR conditions are optimized for the extracted DNA.

Keywords: *Cannabis sativa*,, Secondary metabolite, Polyphenols, PCR amplification, RAPD-PCR, DNA extraction

INTRODUCTION

Over the last two decades, there has been a significant development in the plant molecular biotechnology. Molecular techniques such as RAPD, AFLP, STS and microsatellites have been widely used for genome mapping, genotype studies and identification of associated genes of interest for genetic diversity studies. For facilitation of genomic studies, isolation of high purity DNA is a major pre requisite. It is generally observed that DNA extraction from plants is preferentially initiated from young tissues that have an advantage of comparatively lower content of secondary metabolites such as polysaccharides and polyphenols. Presence of secondary metabolites has been found to hamper DNA extraction

protocols resulting in co precipitation of DNA and therefore inhibiting DNA digestion and PCR[1]. Therefore high purity DNA extraction free from other metabolites is an important aspect and of high significance, making it a determining factor for further genomic studies and molecular mapping. Like many other plants, *Cannabis sativa* L (marijuana), is one of the species with a relatively higher content of polyphenols making DNA extraction a challenging task. *Cannabis sativa* L. (marijuana) is one of the oldest known domesticated plants that is cultivated worldwide owing to its varied usage such as psychoactive cannabinoids, durable fibre, and nutritious seed. Both fibres as well as drug varieties of *Cannabis sativa* L. have been associated with humans since long. The plant (*Cannabis sativa*) is thought to have originated in the central Asia region, and has since been distributed worldwide by humans who have cultivated the plant as a source of fibre, fodder, oils, medicines, and intoxicants for thousands of years [2,3]. Despite cultivation being restricted in many countries due to its psychoactive properties, there has been a resurgence of interest in Cannabis for its agronomic potential, particularly as a source of fibre. Illegal cultivation occurs for the drug marijuana and its derivatives, while naturalized or feral populations are widespread [4]. Since Isolation of pure, intact and high quality DNA is highly crucial for plant genetic studies, special care needs to be taken while DNA isolation of *Cannabis sativa* L where presence of high amounts of polyphenols in plant tissues, may interfere with subsequent DNA manipulation. The presence of polyphenols, which are powerful oxidizing agents present in many plant species, can reduce the yield and purity of extracted DNA [5,6]. The presence of certain metabolites in plant tissues can hamper the DNA isolation procedures and reactions such as DNA restriction, PCR amplification and gene cloning. Problems encountered in the isolation and purification of high molecular weight DNA from certain medicinal and aromatic plant species include; degradation of DNA due to endonucleases, co-isolation of highly viscous polysaccharides, and inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions [7]. A rapid and an effective method of high purity DNA isolation from *Cannabis sativa* L. (marijuana), which is suitable for PCR and mapping has been optimized.

MATERIALS & METHODOLOGY

Plant Material

Juvenile leaves of *Cannabis sativa* were collected from different locations of Haryana, India (Yamunanagar; Longitude 77° 9' 37.188" E, Latitude of 30° 2' 9.2034" N, altitude 260 M above mean sea level; Kurukshetra; Longitude 76° 49' 11.4234" E and Latitude 29° 57' 23.508" N, altitude 260 M mean sea level; Ambala; Longitude $76^0$46'50" E, Latitude of $30^0$22'41" N, altitude 267 M above mean sea level; Kaithal; Longitude 76° 23' 0" E, Latitude of 29° 48' 0" N, altitude 220 M above mean sea level; Karnal; Longitude 76° 59' 0" E, Latitude of 29° 41' 0" N , altitude 228 M above mean sea level). Plant material was preserved in ice bags and DNA was extracted from collected fresh leaves on the same day.

DNA isolation protocol

The DNA isolation protocol is carried out in four steps: extraction from sample using PVP, salt and CTAB; precipitation using high salt concentration; purification and re-precipitation, using isopropanol to remove any left colored polyphenols. Fresh young leaves are collected from different locations, using sterilized forceps. 5 gm leaf material is ground in mortar pestle in liquid nitrogen. After grinding is done, leaf material is transferred to 15 ml microfuge tube and about 7 ml of warm extraction buffer (preheated to 50^0 C in water bath) is added to it. The extraction buffer is of following composition: 200 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2% (w/v) CTAB, 2% (v/v) (β- Mercaptoethanol (added just before use). Also, 500 mg of PVP (100 mg/ gm leaf sample) is added to the tube and it is

vortexed for 2 min. The sample is incubated at 50^0 C for 30 min in water bath with intermittent gentle shaking during incubation. The solution is cooled and 15-20 ml of phenol: chloroform: isoamyl alcohol (25:24:1) is added and mixed by gentle inversions at room temperature for 5-7 min (30-40 times approx.). The tubes are centrifuged at 4000 rpm for 15 min at 28^0 C. Supernatant is taken from the tubes and the above step is repeated to take out sticky residues from the precipitant. Again, supernatant is taken and 15-20 ml of chloroform: isoamyl alcohol (24:1) is added and the solution is mixed by gentle inversions at room temperature for 5-7 min (30-40 times). Then, tubes are centrifuged at 5000 pm for 15 min at 10^0 C. The upper aqueous layer is carefully transferred to another 15 ml microfuge tube. The chloroform: isoamyl alcohol purification process is repeated until the inter-phase becomes clear of any protein content (gelatinous substance). Twice volume of ice-cold isopropanol and half volume of 5M NaCl are added to the supernatant collected and left for overnight at 4^0 C. The DNA is hooked up with sterile Pasture pipette next day and transferred to 1.5 ml eppendorf tube containing 70 % ethanol solution and allowed to fall in tube. The tube is centrifuged for 5 min at 4500 rpm at 4^0 C and supernatant is discarded. Ethanol washing step is repeated twice and the DNA is dried to remove any left traces of ethanol present in it. The DNA is dissolved in 700 µl TE buffer (10 mM Tris-Cl (pH 8.0) and 1 mM EDTA (pH 8.0). To remove any persisting color in DNA sample, it is re-precipitated by adding twice volume of ice-chilled isopropanol and incubated overnight. The ethanol washing is repeated and the DNA pellet is again dissolved into 700 µl TE buffer. Any contamination of RNA is avoided by adding 7 µl of RNase A (0.01 mg/µl) and sample is incubated at 37ºC for 30 min. Re-precipitation step can be repeated with twice volume of ethanol if color still persist. The tubes are centrifuged at 4500 rpm for 5 min at 4^0 C. The pellet is washed with 70% ethanol. The pellet is dried and dissolved in 500 µl of TE buffer or sterile double distilled water and stored at -20ºC until use. DNA obtained with this technique constantly gives 89.5 µg/ml DNA and 260/280 absorbance ratio of 1.8 ± 0.02 indicating high quality intact DNA. This DNA can be stored for months at 4^0 C.

Agarose Gel QC

A 0.8 % (w/v) regular agarose gel in 1X TBE is cast. Placed 5 µL of extracted DNA and 5 µL sterile water in a 0.2 mL microcentrifuge tube along with 2 µL of gel tracking dye. The gel is run for 30 min. at 100v. Gel is visualised in UV transilluminator. Absence of any smear below bands or sheared bands in gel, shows absence of RNA and intact DNA (Fig 1).

PCR Amplification of Extracted DNA

The polymerase chain reaction (PCR) is an efficient and quick *in vitro* method for enzymatic amplification of DNA sequences. A simple PCR reaction consisting of a set of synthetic primers (oligonucleotide) that flanks the target DNA sequence, a DNA polymerase and dNTPs is carried out in various cycles. Repetitive cycles are carried out that involve template denaturation, primer annealing and extension; yielding tremendous amount of DNA. To amplify extracted DNA for further analysis and for the optimization of RAPD reaction using DNA extracted from Cannabis plant species, oligonucleotide primers (BangloreGenei Banglore, India) were used for amplification for standardized PCR conditions. The reactions were carried out in a Thermocycler. Reactions without DNA served as negative controls. Optimization of PCR condition was achieved by applying variations in genomic DNA concentration (ng), deoxynucleotide triphosphates (dNTP's), primer concentration (µM), $MgCl_2$ conc. (mM), Tris-HCl (mM) used in *Taq*. buffer, *Taq.* polymerase (U/ µl). Also, time and temperature of initial denaturation step, time for denaturation, time and temperature for annealing, extension period and final extension period along with the number of cycles were varied under a set of different ranges. The reaction was stopped by chilling at 4^0 C. Two

RAPD primers, 6800-042 (5'-ACCCAGGTTG-3') and 6800-043 (5'-GTGACCAGAG-3') (decamers) were basically used for PCR reaction (Bangloregenei, Banglore). The 20 µL PCR reaction volume per tube contained 50 ng DNA, 10 X *Taq* buffer mix (750 mM Tris-HCl, pH 8.3), 1.5 mM $MgCl_2$, 0.75 µM of RAPD primer (10 µM), 0.75 mM of dNTP's mix (80 mM stock) and 1.5 U *Taq* DNA Polymerase (Genaxy, 1U/µl). The reaction profile comprised 5 min incubation at 94 °C for initial denaturation and then a cycle of 94° C for 1 min, 42^0 C for 1 min and 72° C for 2 min, repeated 35 times. Following cycling, the reaction was held at 72 °C for 10 min, before a final 4 °C holding temperature. PCR products were subjected to electrophoresis on 1.5 % (w/v) agarose gels, in 1X TBE Buffer at 50 V for 3-4 h until the bands separate completely and then stained with ethidium bromide (0.5 µg/ml). Gels with amplification fragments were visualized and photographed under UV light (Figure 2 and Figure 3).

RESULTS AND DISCUSSIONS

There are number of challenges in isolation and purification of DNA especially from medicinal and aromatic plants; that includes degradation of DNA due to endonucleases, coisolation of highly viscous polysaccharides, inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions[8] . Moreover, in case of isolation of genomic DNA from medicinal plants such as *C. sativa* isolation of high purity DNA becomes tedious task owing to presence of polyphenols and polysaccharides in large number. During isolation procedure polysaccharides are found to form complexes with nucleic acids forming a gelatinous mass, thereby physically inhibiting the DNA from the action of DNA modifying enzymes *e.g.* restriction enzymes, DNA polymerase, ligase, etc.[9] . Although a number of protocols for DNA isolation from plants containing high amount of secondary metabolites have been developed and published[6,8,9,10,11,12] in all the cases, low concentrations of polyphenols and polysaccharides were found to be obtained at the end, leading to sticky, viscous and colored DNA that is not suitable for PCR amplification. To overcome this problem, a novel protocol of DNA extraction has been presented, with high concentration of CTAB. Similar concentration was used for various medicinal and aromatic plants previously as well [6,13,14]. High ionic strength of CTAB forms complexes with proteins and most of the acidic polysaccharides. The problems created by the presence of phenolic compounds can be reduced by the addition of 1% (or higher) polyvinylpyrollidine (PVP) in the initial isolation buffer, just before the incubation in water bath. PVP was used at a concentration of 100mg/ gm of leaf sample. In this study, we used the different concentrations of CTAB, PVP and NaCl. In our protocol, the polyphenols find their way in DNA preparation during the liquid nitrogen homogenization process. High concentration of β-mercaptoethanol was helpful in removing tannins and polyphenols efficiently from the DNA samples [15]. The salt, NaCl is needed for the formation of a nucleic acid precipitate, for this NaCl at elevated concentration (> 0.8 M NaCl) was used in DNA isolation protocol. High concentration of NaCl (5 M) was used along with ice-chilled isopropanaol in the DNA precipitation step in the last stage to remove yellowish/ brownish color of the DNA pellet which was because of the polysaccharides. Re-precipitation was performed on the DNA dissolved in TE by using twice volume of ice-chilled isopropanol and incubated for overnight this further removed from it, any traces of secondary metabolites [6, 16] . The polysaccharides were removed using extraction buffer containing high NaCl concentration. This protocol gives DNA of high purity and free from any secondary metabolites, from *C. sativa* (Figure 1). The DNA purity and concentration is checked by running isolated DNA samples on 0.8% agarose gel and by taking absorbance at 260 nm in UV/VIS spectrophotometer. DNA purity was

determined by calculating the absorbance ratio A260/280. A sufficient amount of clean genomic DNA was obtained with this modified method. The yield was about 86.5 µg/ml and the A260/280 nm ratio was 1.804 ± 0.031. Isolated DNA is used for RAPD-PCR with two primers and it gave good results (Figure 2 and Figure 3) with both the primers. The process uses three major modifications to the existing techniques: precipitation using both polyvinylpyrrolidone (PVP) and high salt, and phenol: chloroform purification and reprecipitation using twice volume of isopropanol and high salt. Present method introduced through this paper is simple and reliable for the isolation of high purity and reproducible genomic DNA from *C. sativa*, overcoming difficulties owing to high amount of polyphenolic or polysaccharide components. A substantial amount of reproducible DNA from each sample can be easily isolated and used directly for PCR amplification. The isolated DNA is found to be good for PCR amplification using RAPD primers and in future prospects can prove vital for forthcoming molecular and genetic studies.

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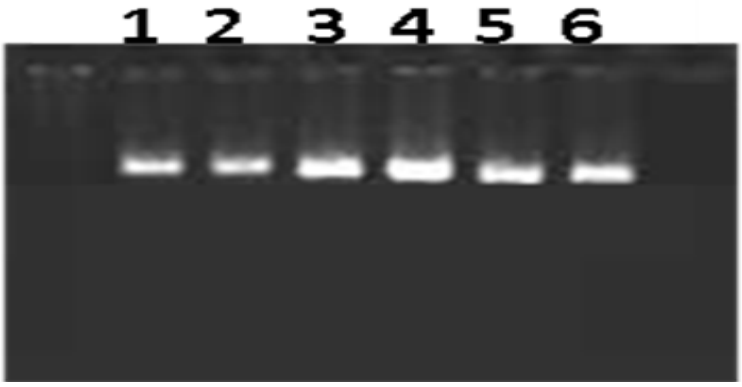

Figure 1: Lanes 1-6 represents the agarose gel (0.8 %) of DNA isolated from *Cannabis sativa* from different locations of Haryana. Lane 1 and 2 are the samples from Kurukshetra (different locations), lane 3,4,5 and 6 are the samples from Yamunanagar, Ambala, Karnal and Kaithal; respectively.

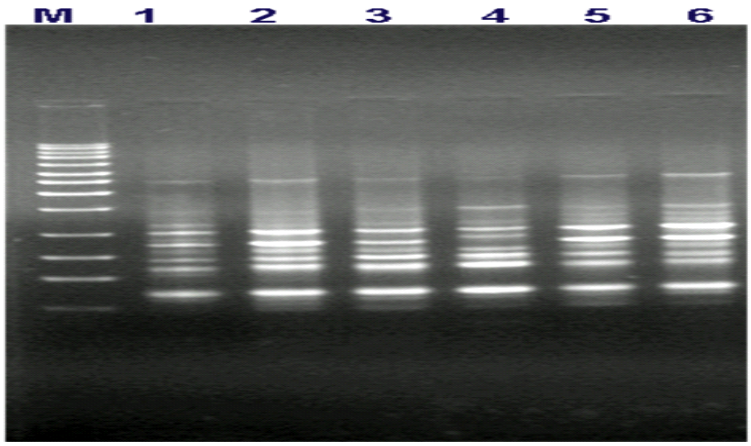

Figure 2: Amplification of purified DNA with RAPD-PCR. DNA was purified using the method described. The amplification products were separated on a 1.5% agarose gel, stained with ethidium bromide and visualised with UV light. Lane M contains a 100 bp DNA size marker. Lanes 1 and 2 are *Cannabis sativa* samples from Kurukshetra and lanes 3,4,5 and 6 represents *Cannabis sativa* samples collected from Yamunanagar, Ambala, Karnal and Kaithal; respectively and are amplified using 6800-042 primer (5'-ACCCAGGTTG-3').

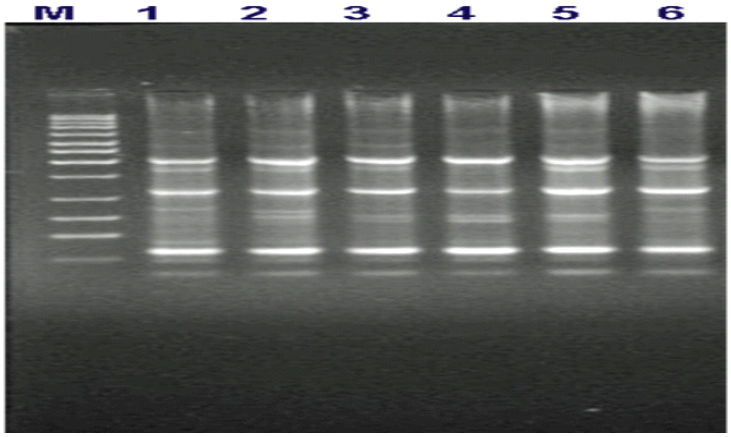

Figure 3: Amplification of purified DNA with RAPD-PCR. DNA was purified using the method described. The amplification products were separated on a 1.5% agarose gel, stained with ethidium bromide and visualised with UV light. Lane M contains a 100 bp DNA size marker. Lanes 1 and 2 are *Cannabis sativa* samples from Kurukshetra and lanes 3,4,5 and 6 represents *Cannabis sativa* samples collected from Yamunanagar, Ambala, Karnal and Kaithal; respectively and are amplified using 6800-043 primer (5'- GTGACCAGAG-3').