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Development of Liquid Nitrogen Free and Efficient DNA Isolation Method for Plants Rich in Volatile Oil, Secondary Metabolites and Polysaccharides

Manish Kumar Suthar*, V. Thondaiman, Parmeshwar Lal Saran and Manish Kumar Mittal

ICAR-Directorate of Medicinal and Aromatic Plants Research, Anand, Gujarat-387310, India

**Corresponding author*

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A B S T R A C T

Ocimum sanctum L. or "Tulsi" or Holy basil is a plant well known for its medicinal properties. This plant is rich in essential oil sequestered in peltate glands. The Tulsi is rich source of eugenol, methyl eugenol, chavicol, ursolic acid, rosmerinic acid and other phenolics and terpenes. *Aloe barbadensis* and *Plantago ovata* are plants rich in polysaccharides. These plants are very important medicinal plants. *A. barbadensis* is source of aloin which is used in various cosmetic products and *P. ovata* is the main source of Isabgol-Husk. DNA based studies in these and similar plants require optimization of DNA isolation method due to presence of high level of secondary metabolites and polysaccharides. Which generally hamper all downstream enzymatic analysis like PCR amplification. DNA isolated using standard DNA isolation method especially from stored leaves from such plants is not suitable for downstream enzymatic analysis like PCR. In present study, a DNA isolation method has been developed which results in high quality and intact DNA from Tulsi, *A. barbadensis* and *P. ovata*. This method is free from the use of liquid nitrogen, Phenol, and Cetyltrimethyl ammonium bromide and moreover efficient to isolate intact DNA which can be easily used for downstream molecular analysis. DNA isolated by newly developed method was successfully amplified by PCR reaction using random and specific primers.

Introduction

Isolation of pure and high-quality DNA from plants is crucial step towards molecular biological studies. DNA isolation process requires costly reagents and instrumentation. However, high levels of secondary metabolites and polysaccharides affect the purity and quality of isolated DNA. Generally, the DNA isolation protocol is to be developed or modified according to different plant species having different metabolites. Holy basil or *Ocimum sanctum* L. (*O. tenuiflorum*) is a sacred plant of India and belongs to

Lamiaceae family. This herb is also known as "Tulsi", "The Queen of herbs" and "The Mother Medicine of Nature" (Singh and Hoette, 2002). *O. sanctum* has been indicated as anti-microbial, anti-spasmodic, anti-diabetic, anti-cancer and anti-fertility agent (Pattanayak *et al.,* 2010). The chemical composition of different varieties or accessions of *O. sanctum* is very complex having various phytochemicals in different proportions.

All herbage parts of this species have high level of secondary metabolites. Specifically, the leaves have peltate glands and other glandular structures which synthesise volatile oils containing eugenol, chavicol, methyl eugenol, ursolic acid, caryophyllene, methyl chavicoletc (Kelm *et al.,* 2010; Shishodia *et al.,* 2003; Iijima *et al.,* 2004). These metabolites affects the quality of DNA isolated from *O. sanctum*. At present, commercially available kits for DNA isolation or purifications seems the only source for high quality DNA purification. This is also evident in the genomic DNA isolation methodologies used for *O. sanctum* in recent reports (Rastogi *et al.,* 2015; Upadhyay *et al.,* 2015). But use of such kits increases cost of studies. Similarly, DNA isolation from some plant species rich in polysaccharide like *Aloe barbadensis* and *Plantago ovata* using CTAB methods and commercially kits is very difficult. Such species give sticky DNA samples which cannot be used for PCR amplifications. There are no single DNA isolation method which can universally be used to isolate DNA from *O. sanctum*, *A. barbadensis* and *P. ovata*. In present study, we have developed a methodology for the isolation of high-quality DNA from *O. sanctum, A. barbadensis and P. ovata* amenable to be used in further downstream molecular studies. This newly developed methodology is a cost effective as it eliminates use of CTAB, phenol and liquid nitrogen, rapid and also equally effective in fresh as well as stored leaves. This methodology may be useful in other plant species having high level of contaminating metabolites and polysaccharides in affordable manner.

Materials and Methods

Plant samples and DNA isolation

Four different accessions of *O. sanctum* species were used to isolate the DNA (Fig. 1). These different accessions have different degree of phytochemical present. Some accessions have comparative high level of volatile oils and peltate gland and some are known to have different terpenes and phenylpropanoids. These plants were grown in field and leaves were harvested and stored for one year in minus 80 deep freezer. Further, DNA was also isolated from two polysaccharide rich crops *Aloe barbadensis* and *Plantago ovate*.

Modified CTAB method: This method was based on method given by Doyle and Doyle (1990) with some modification.

300-400 mg leaf sample was ground in liquid nitrogen in 800 µl of 4% CTAB extraction buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA with 4% CTAB) containing PVP (up 2%).

Samples were incubated at 64 °C for 1 hr with mixing after each 15 min. intervals.

Centrifuged at 12,000 × g for 5 min. and supernatant was collected.

RNaseA was added and incubated at 37 °C for 20 min.

Equal volume of chloroform:isoamylalcohol (C:I) (24:1) or P:C:I (25:24:1) was added.

Emulsions were centrifuged for 15 min. at 14,000 × g.
The aqueous supernatants were transferred to separate tubes and repeated the same step.

In aqueous supernatants, ice cold isopropanol was added and mixed gently.

Incubation at minus 20 °C was given overnight.

Samples were centrifuged for 14,000 × g for 10 min.

Pellets were washed with 70 % alcohol by spinning at 12,000 × g for 8 min.

The pellets were air dried until complete loss of alcohol smell. Finally, air dried pellets were suspended in molecular biology grade water. CTAB method followed by purification by Kit: DNA isolated by above method was taken as starting material for the kit supplied by thermo scientific (GeneJET Plant Genomic DNA Purification Kit). Finally, DNA was eluted in elution solution supplied with kit.

Our Methods

Prepare extraction buffer (sucrose (0.5 M), Tris (150 mM), NaCl (1 M), EDTA (40 mM), TritonX100 (2%) and BME (2.5%).

Grind 200-300 mg leaves samples in extraction buffer (having a pinch of PVP).

Incubate suspended material at 60 °C for 30 min.

Centrifuge at 12,000 × g for 10 min and discard supernatants.

Suspend the pellets in lysis buffer contain 2 % SDS, 120 mM Tris, 20 mM EDTA, 1.4 M NaCl and 1% BME.

Mix the suspension and incubate at 64 °C for 45 min.
Centrifuge for 12,000 × g for 12 min.

Collect the supernatants and add equal amount of C:I.

Mix the sample to form emulsion and centrifuge for 14,000 × g for 15 min.

Collect upper clear aqueous layer and add 1/10 volume of 3 M sodium acetate (pH 5.2).

After mixing, add 2/3 volume of ice-cold isopropanol and mix gently.

Incubate at minus 20 °C for 1 hr.

Centrifuge at 14,000 × gfor 10 min.

Wash the pellets with 70 % alcohol solution by centrifuging at 12,000 × g for 8 min.

Air dry the pellets and dissolve in molecular grade water.

These DNA solutions were directly used in PCR or RNA was removed as described above. Genomic DNA samples were analysed using 0.8 % agarose gel electrophoresis. Quantitative estimations of all DNA samples were performed by Nano Drop spectrophotometer.

PCR amplification

DNA isolated was subjected to PCR amplification. 50 ng DNA was PCR amplified using both gene specific primers and primers developed for SSR in *O. sanctum* (unpublished). For gene specific primer amplification, the PCR cycles were as: 94 °C for 4 min., 35 cycles of 94 °C for 45 sec., 54.5 °C for 45 sec., 72 °C for 55 sec., followed by final extension of 72 °C for 7 min. For SSR primers the annealing temperature was 53 °C

and remaining protocols were same with minor variations. *A. barabadensis* and *P. ovata* DNA isolated using newly developed method was amplified using primers designed for SRAP marker analysis (primers: Me1+Em6 and Me2 +Em6) and PCR cycles defined for SRAP analysis (Bhatt *et al.*, 2017). PCR amplified products were analysed by 1.2 % agarose gel with ethidium bromide.

Results and Discussion

Pure and high-quality DNA is starting point for any DNA based molecular study. Secondary metabolites form complexes with DNA which hinders the downstream enzymatic reactions. The use of CTAB and liquid nitrogen generally has their own limitations. CTAB method requires fresh preparations each time as CTAB buffer cannot be stored for long time. Second, liquid nitrogen is not available easily in developing countries but these are main hot spots for medicinal plants. Phenol also associated with its own corrosive nature. Overall, there is always a need to develop a method which is free from all above limitations. Agarose gel electrophoresis of isolated DNA from *Ocimum sanctum*is shown in Figure 2a. Intensity of bands and absence of smearing indicated that sufficient and intact DNA was isolated using all three methods used in present study. Table 1 shows quantitative estimation of isolated DNA samples. The quantitative estimation suggested that all used methods seem equally efficient to isolate DNA from the accessions of different chemical compositions. After diluting the *O. sanctum* DNA samples (50 ng/µl) PCR amplifications were carried out and results of amplifications are shown in Figure 2b. Interestingly, despite the good quantity and intact nature all DNA isolated from all methods used, DNA isolated from *O. sanctum* using CTAB alone failed to amplify gene specific and SSR primers. But DNA isolated by CTAB method followed by purification by kits resulted in to amplifications. These results indicated that some interfering metabolites may be present in DNA which may form complex with DNA and limits the enzymatic reactions. Even after the purification with P:C:I isolated DNA failed to give PCR amplification. Use of kits for further purification of DNA may eliminate these interfering molecules. But contrasting results were obtained when DNA was isolated with SDS based methods. Regardless of chemically different accessions used in the study our new method resulted in to amplification in all samples.

Table.1 DNA quantitative and qualitative analysis

Plants	CTAB method		CTAB followed by Kit		Newly developed Method	
	260/280 ratio	Yield (µg/g of plant)	260/280 ratio	Yield (µg/g plant)	260/280 ratio	Yield (µg/g plant)
***O. sanctum* accession 1**	1.78	255	1.85	52	1.89	110
***O. sanctum* accession 2**	1.76	285	1.86	64.5	1.90	135
***O. sanctum* accession 3**	1.69	205	1.84	59	1.87	115
***O. sanctum* accession 4**	1.81	265	1.88	75	1.88	145
Aloe barbadensis	1.81	135	-	-	1.91	130
Plantago ovata	1.84	155	-	-	1.90	155

Fig.1 Abaxial and adaxial surfaces of different accessions of *O. sanctum* used in present study. A. Rich in peltate glands, B: Lowest in peltate gland, C: High phenolic content and D: High pigment content

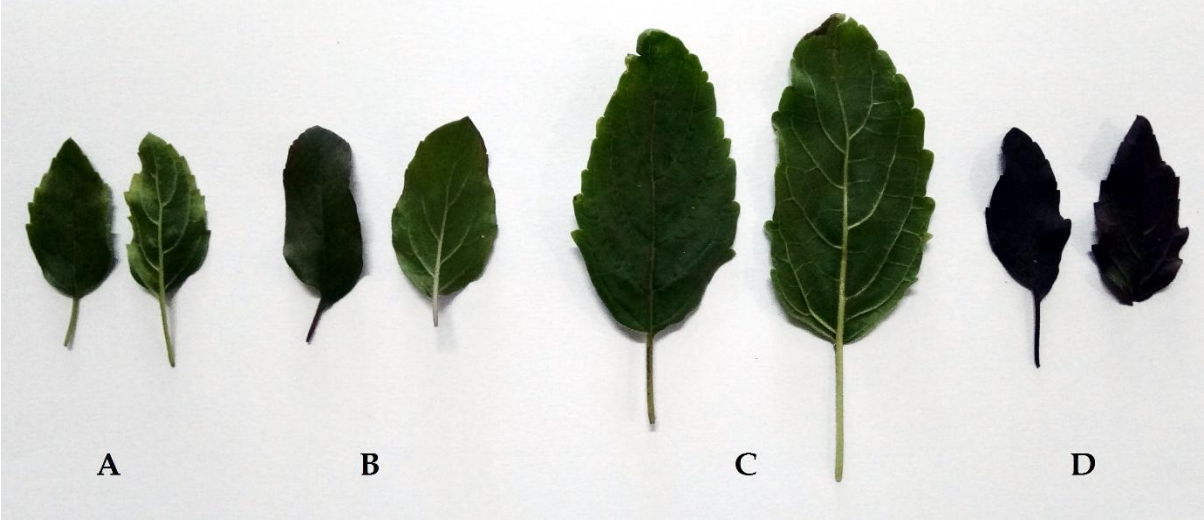


Fig.2 A. 0.8 % agarose gel electrophoresis of isolated DNA from *O. sanctum* samples. A: DNA isolated using CTAB method, B: DNA isolated using CTAB method and purified by Kit, C: DNA isolated newly developed method. b. 1.2 % agarose analysis of PCR amplified DNA of *O. sanctum*. A: PCR amplification of DNA isolated by CTAB method with gene SSR primers (lane 1 to 4) and gene specific primers (lane 5 to 8), B: PCR amplification (SSR primers) of DNA isolated by CTAB method and purified by kit (lane 1 to 4) and newly developed method (lane 5 to 8), C: PCR amplification of DNA isolated by newly developed method and gene specific primers. M represents 100 bp DNA ladder

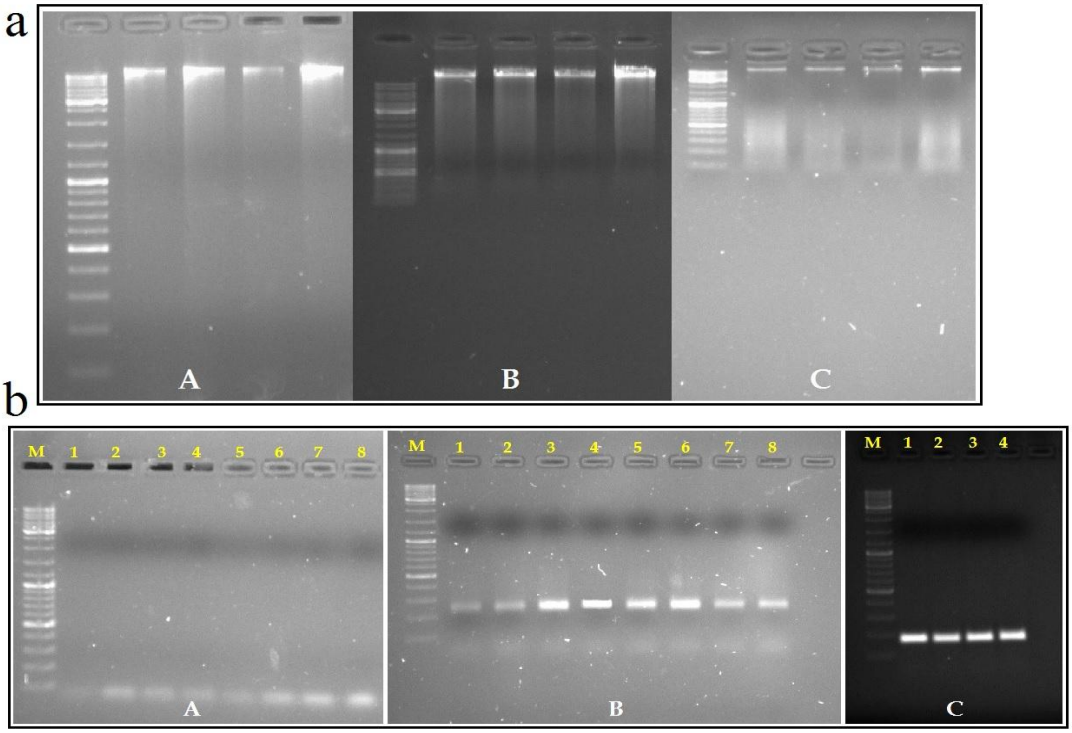
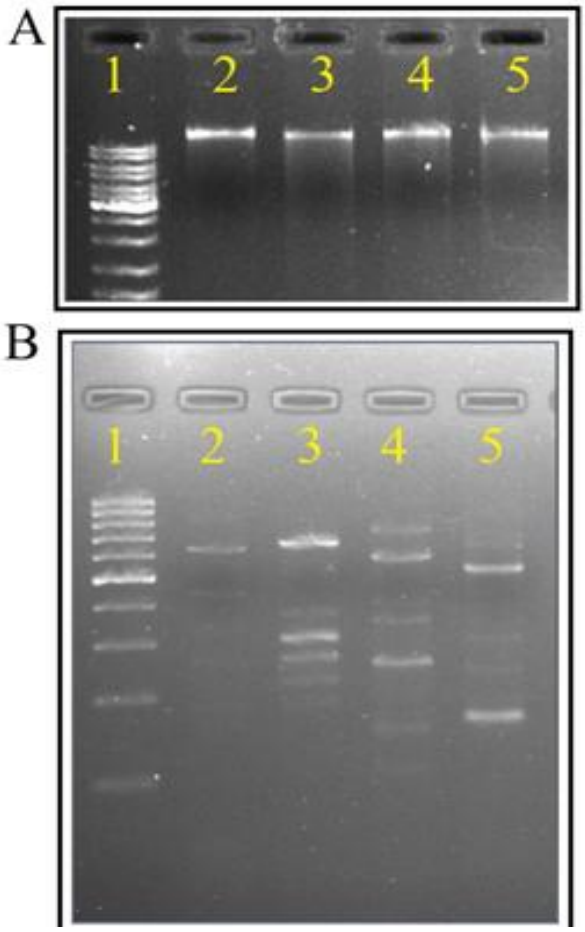


Fig.3 A. 0.8 % agarose gel analysis of *A. barbadensis* and *P. ovata* DNA. Lane 1 represents DNA ladder (100 bp), lane 2 and 3 show DNA isolated from using CTAB method from *A. barbadensis* and *P. ovata* respectively and lane 4 and 5 show DNA isolated using newly developed method from *A. barbadensis* and *P. ovata* respectively. B. 1.2 % agarose electrophoresis of PCR amplified DNA of *A. barbadensis* and *P. ovata* DNA using SRAP markers. Lane 1 shows 100 bp DNA ladder, lane 2 and 3 show Me1+Em6 amplification from *A. barbadensis* and *P. ovata* respectively and lane 4 and 5 show Me2+Em6 amplification from *A. barbadensis* and *P. ovata* respectively



The newly developed method is cost effective, rapid and efficient method for the intact and high-quality DNA isolation from the plants which are rich in volatile oils and secondary metabolites. This method does not require CTAB, costly liquid nitrogen and hazardous phenol. Also, above method is equally effective for plant leaves having high degree of polysaccharides.

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