Optimization of DNA extraction protocol for polymerase chain reaction based methods in phenolic and polysaccharide rich Sesamum indicum L.

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Abstract

Molecular studies of plants rely on isolation of pure and high molecular weight genomic DNA. The chemotypic heterogeneity among species may not permit optimal DNA yields with a single protocol, and thus, even closely related species may require different isolation protocols. *Sesamum*, an oil yielding crop, contains high levels of polyphenols, polysaccharides and secondary metabolites. These metabolites can interfere with the DNA isolation methods affecting genomic DNA isolation and other downstream reactions such as restriction digestion, PCR etc. In this study, a simple, rapid and efficient method for leaf DNA extraction with slight modifications of CTAB method in *Sesamum* is optimized. The procedure involves homogenization of the plant leaf in extraction buffer, incubation at 60ºC, and extraction by different fixing solutions containing absolute alcohol, chloroform and EDTA without liquid nitrogen. The results showed that the extracted DNA quality and quantity were comparable to those isolated with liquid nitrogen as the $\lambda_{260}/\lambda_{280}$ ratio with liquid nitrogen was in the range 0.5-1.74 and with other fixing solutions it was 0.28-1.36. Absolute alcohol showed best results as fixing solution. The extracted DNA can be used directly for PCR.

Keywords: CTAB, DNA isolation, PCR, polyphenols, polysaccharides**.**

Abbreviations:

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CTAB: Cetyltrimethylammonium bromide

SDS: Sodium dodecyl sulphate

PCR: Polymerase chain reaction

PVP: Polyvinylpyrrolidone

EDTA: Ethylenediaminetetraacetic acid

NaCl: Sodium chloride

EcoRI: *Escherichia coli* RI

OD: Optical density

dNTPs: Deoxynucleotide triphosphates

RAPD: Random Amplification of Polymorphic DNA

Introduction

Sesame (*Sesamum indicum* L.), is one of the most ancient oil seed crops known to mankind. Sesame seed is highly nutritive as it contains oil (44–58%), protein (18–25%), carbohydrate (~13.5%) and ash (~5%) (Yoshida, 1994; Mohamed and Awatif, 1998; Shyu and Hwang, 2002; Kahyaoglu and Kaya, 2006) and its oil contains an antioxidant called sesamol which imparts it a high degree of resistance against oxidative rancidity (Ashri 1989; Abou-Gharbia et al. 2000). It is also a rich source of lignans, polyphenols, carbohydrates and minerals. It is used in the production of oil, paste (tehineh) and in food formulations such as Halaweh (sweetened tehineh), java beans and salads (Namiki 1995; Abu-Jdayil et al., 2002). Sesame seeds yield is low due to lack of improved cultivars. Its production is also limited by pests, diseases, lack of uniform maturity of capsules, and seed shattering. For any improved production of sesame using molecular tools, extraction of pure and high quality DNA is required. However, it is difficult to isolate high-quality DNA from *Sesamum indicum* due to the presence of high amount of polyphenols,

polysaccharides, essential oil and other secondary metabolites. In addition, these contaminants interfere in downstream reactions such as DNA restriction, amplification and cloning. A good extraction procedure for the isolation of DNA should yield adequate and intact DNA of reasonable purity. The procedure should also be quick, simple and cheap and, if possible, avoid the use of dangerous chemicals. The first step in the extraction process involves digesting away cell wall in order to release the cellular constituents. For this usually an initial grinding with liquid nitrogen is employed. But liquid nitrogen is costly and may also be difficult to procure in remote areas. This can be prevented by fixing leaves in different solutions before DNA extraction, without liquid nitrogen (Sharma et al., 2010). This is followed by disruption of the cell membrane to release the DNA into the extraction buffer. This is normally achieved by using detergents such as sodium dodecyl sulphate (SDS) or cetyltrimethylammonium bromide (CTAB). The released DNA should be protected from endogenous nuclease. EDTA is often included in the extraction buffer to chelate magnesium ions, a necessary co-factor for such nucleases. The initial DNA extracts often contain a large amount of RNA, proteins, polysaccharides, tannins and pigments which may interfere with the extracted DNA and make it difficult to separate. Most proteins are removed by denaturation and precipitation from the extract using chloroform and/or phenol. RNAs on the other hand are normally removed by treatment of the extract with heat treated RNase A. Polysaccharide-like contaminants are, however, more difficult to remove. They can inhibit the activity of certain DNA-modifying enzymes and may also interfere in the quantification of nucleic acids by spectrophotometric method (Wilkie et al., 1993). NaCl at concentration of more than 0.5 M, together with CTAB is known to remove polysaccharides (Murray and Thompson, 1980; Paterson et al., 1993). The concentration ranges mentioned in literature vary between 0.7 M (Clark, 1997) and 6 M (Aljanabi et al., 1999) and are dependent on the plant species under investigation.

The chemotypic heterogeneity among species may not permit optimal DNA yield from one isolation protocol, and perhaps even closely related species may require different isolation protocols (Weishing et al., 1995). A number of methods are available for extraction of nucleic acids from the plants (Dellaporta et al., 1985; Saghai-Maroof et al., 1984; Doyle and Doyle, 1987). In this study three modifications in DNA extraction were carried out in *Sesamum indicum* L. Modifications were made to minimize polysaccharide co-precipitation and complete removal of proteins and polyphenols.

Material and methods

Plant Material

Seeds of *Sesamum indicum* L. (RT-125) were collected from Krishi Vigyan Kendra, Banasthali, Tonk, Rajasthan, India. Seeds were surface sterilized in 0.1% mercuric chloride and sown in heat sterilized soil in small plastic plots. And pots were kept in plant growth chamber at 25-27°C in light for 12 hours. For experiment, 14 days old plant's leaves were taken.

DNA isolation

Fresh leaves of plant were dipped in different fixing solutions of various combinations to isolate DNA. This treatment was given to denature enzymes at room temperature for 40 min. Various fixing solutions used were absolute alcohol (60%, 70% and 80%), alcohol: chloroform and alcohol: EDTA in varying ratios (60:40, 70:30 and 80:20). Other modifications were also used for e.g. polyvinylpyrrolidone (PVP) (1%, 2 and 4%), β-mercaptoethanol (1% and 2%), CTAB (2% and 5%) and SDS (2% and 20%). Variation was also done in incubation time from10 min to 60 min at 60ºC and centrifugation speed from 12000 rpm to 10000 rpm. After that extraction was done with chloroform: isoamylalcohol (24:1) and supernatant was decanted in another tube. Now, precipitation was done with equal volume of isoamylalcohol as of supernatant volume. The precipitated DNA was collected and washed thrice with 70% ethanol. Finally pellet was air-dried and dissolved in TE buffer and RNase treatment was given at 37°C.

Quantification of Genomic DNA

The DNA was quantified by taking optical density (OD) at λ_{260} nm and λ_{280} nm using a Spectrophotometer (Systronic UV-visible). DNA purity was determined by calculating the absorbance ratio $\lambda_{260}/\lambda_{280.}$ The DNA sample was also quantified on 0.8% agarose gel electrophoresis. For this, 0.8 gram agarose was dissolved in 100 ml of 0.5X TBE buffer of pH 8.0 (Tris base, boric acid, EDTA). TBE is generally made and stored as 5X stock solution (54 gram Tris base, 27.5 gram boric acid and 20 ml 0f 0.5 M EDTA were dissolved in 1 liter distilled water) (Sambrook et al., 1989). And heated in microwave oven at 90-95 °C until gel melt. Now, the gel is cooled to 60 °C and ethidium bromide (final concentration of 0.5 µg/ml) was

added for visualization of bands under UV light and then melted gel was poured in casting tray containing combs. After solidification at room temperature, tray was mounted in electrophoresis buffer tank containing 0.5 X TBE buffer and combs were removed. Now, 5 µl DNA was mixed with 2 µl of 6X loading buffer (0.25% bromophenolblue, 0.25% xylene cyanol and 30% (w/v) glycerol in distilled water) and loaded into the well made by comb (Sambrook et al., 1989). And run gel at voltage of 1-5 V/cm and run until the gel run sufficient distance as indicated by dye. And for comparison λ DNA EcoRI/Hind III double digested was loaded.

PCR amplification

RAPD analysis of genomic DNA was done with nine random primers (Table 1). PCR mixture (25µl) contained Taq buffer (2.5 µl), dNTPs (2.5mM), primers (10 µM), Taq polymerase (3 U) and DNA (50 ng). The thermal cycler was operated- 1 cycle at 94ºC for 4 min; 44 cycles at 94ºC for 1 min, 37ºC for 1 min and 72ºC for 5 min; and a final extension at 72ºC for 7 min. Amplified fragments were separated on 1.2% agarose gel (1.2 gram agarose in 100 ml TBE buffer) containing ethidium bromide (0.5 µg/ml).

Restriction analysis of DNA

DNA was restricted by EcoRI (Bangalore Genei) using 3 U/µg DNA. The reaction was incubated at 37ºC for 2 hr. Digested DNA was analysed on 0.8% agarose gel, stained with ethidium bromide (0.5 µg/ml) and observed under UV light.

Results and Discussion:

Sesamum is rich in polyphenols, polysaccharides and oil which make DNA isolation difficult. A number of protocols and their modifications were developed and employed to isolate quality DNA from different plant species in the past (Murray and Thompson, 1980; Dellaporta et al., 1983; Saghai-Maroof et al., 1984). In the present study, plant leaves were fixed in different fixing solutions (absolute alcohol, alcohol: chloroform and alcohol: EDTA) for genomic DNA isolation. Alcohol: EDTA was proved good fixing agent. In addition, leaves were also homogenized in liquid nitrogen for comparison which generally yields rich quality DNA due to fine grinding as improved breakage of cell membrane (Toader et al., 2009). It also

protect DNA against degradation as DNase is inactivated at low temperature (-196°C). Bands of different intensities were visualized while observing agarose gel under UV- rays which indicate different level of protein and polysaccharides impurities in isolated DNA (Fig. 1, 2 and 3). Bright bands were obtained in the initial two modifications but in case of 3rd modification, bands appeared only with alcohol: EDTA. The $\lambda_{260}/\lambda_{280}$ ratio which shows purity of DNA was in the range of 0.5-1.74 with liquid nitrogen and it varied from 0.28-1.36 with other fixing solutions. The DNA concentration was found 95-1180 ng/µl with liquid nitrogen and 65-1360 ng/µl without liquid nitrogen. This showed that DNA extracted without liquid nitrogen was comparable with liquid nitrogen method. Several other modifications in extraction buffer were used for DNA isolation, with one parameter tested at a time to address the problem of phenolics. Modifications included use of different concentrations of β-mercaptoethanol (1-2%), PVP at different concentrations (1-4%) and increased concentration of CTAB. All modifications yield high quality DNA but use of 2% β-mercaptoethanol, 4% PVP and 5% CTAB was found to be most appropriate (Table 2). It makes its use more economical especially in laboratories where availability of liquid nitrogen is a limiting factor. High concentration of PVP was used to remove polyphenols as it form hydrogen bonds in presence of chloroform (Maliyakal, 1992; Haaf et al., 1985). CTAB form an insoluble complex (Murray and Thompson, 1980) which is separated from the residual soluble proteins, polysaccharides and other molecules by centrifugation. β-mercaptoethanol break disulfide bonds of proteins and chloroform:isoamylalcohol treatment was required for removal of major proteins, phenolic compounds and cell debris (Murray and Thompson, 1980; Zhang and Stewart, 2000). And the DNA extracted by fixing in alcohol: EDTA and liquid nitrogen by 3 modifications was used further for amplification and restriction digestion. Moreover, the procedure also eliminated the necessity of phenol, which makes the method less hazardous. PCR amplification was done with nine random primers, out of two primers (RP-1 and RP-2) showed good amplification as shown in fig. 4. As it was random amplification, so the number of bands obtained was large (1-9). It indicated that good quality DNA was obtained by different modifications (Fig. 4). And when genomic DNA was restricted with EcoRI (incubated at 37 ºC for 2 hr, complete smear obtained in entire lanes which indicate that digestion had occurred successfully and no polyphenols or other contaminants were present in DNA (Fig. 5). Because no restriction occurred if polyphenols and polysaccharides present bands remain intact. In general, overall these modifications allowed cheaper, simple and high yielding

methods of DNA isolation. And these methods of genomic DNA extraction will be useful for molecular, genetic diversity and transgenic studies in *Sesamum indicum* L and will be economical too.

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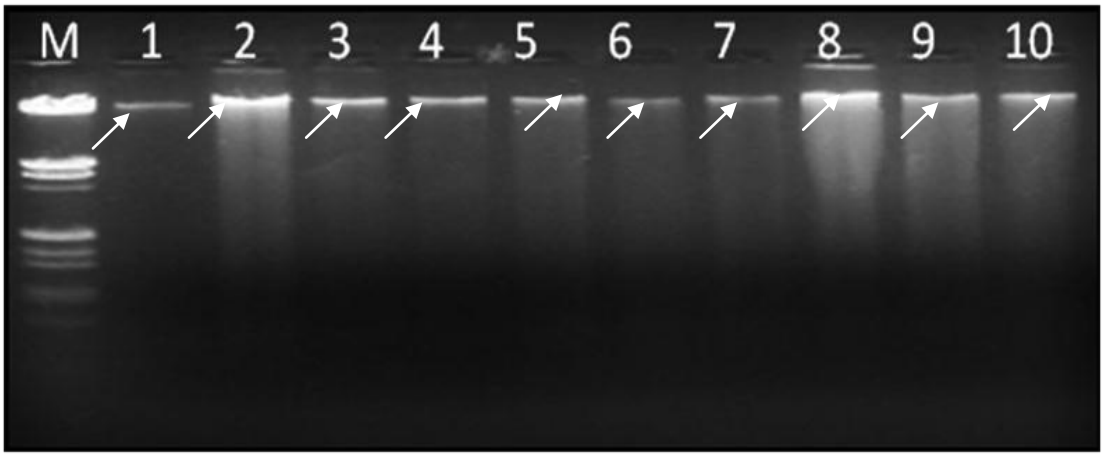
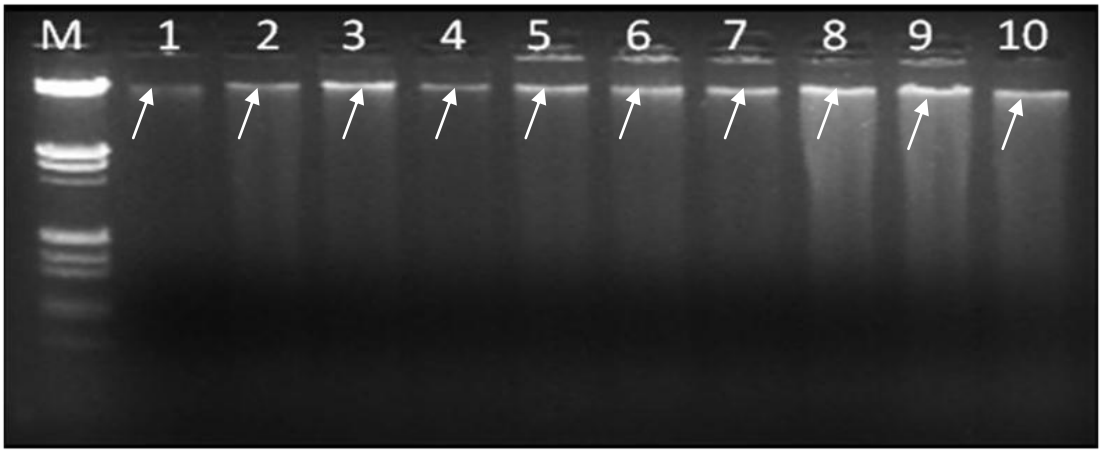


Figure 1.



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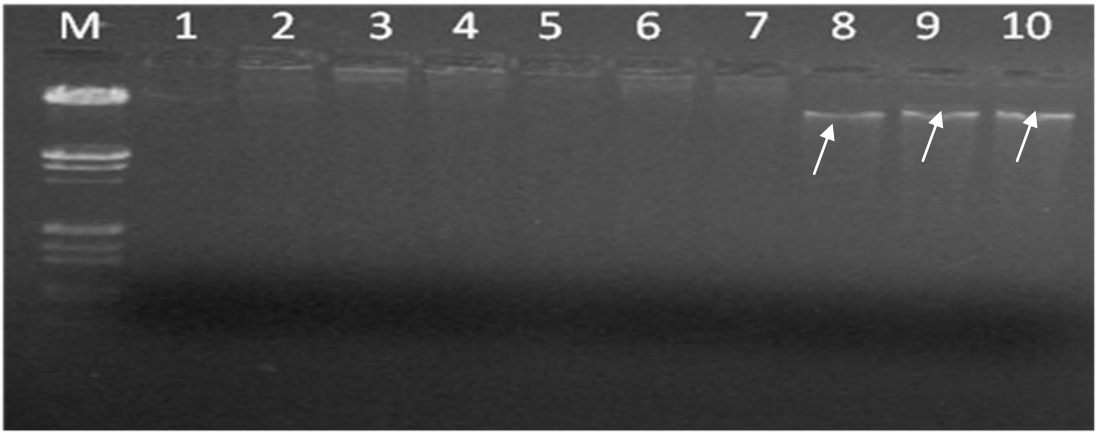

Figure 3.

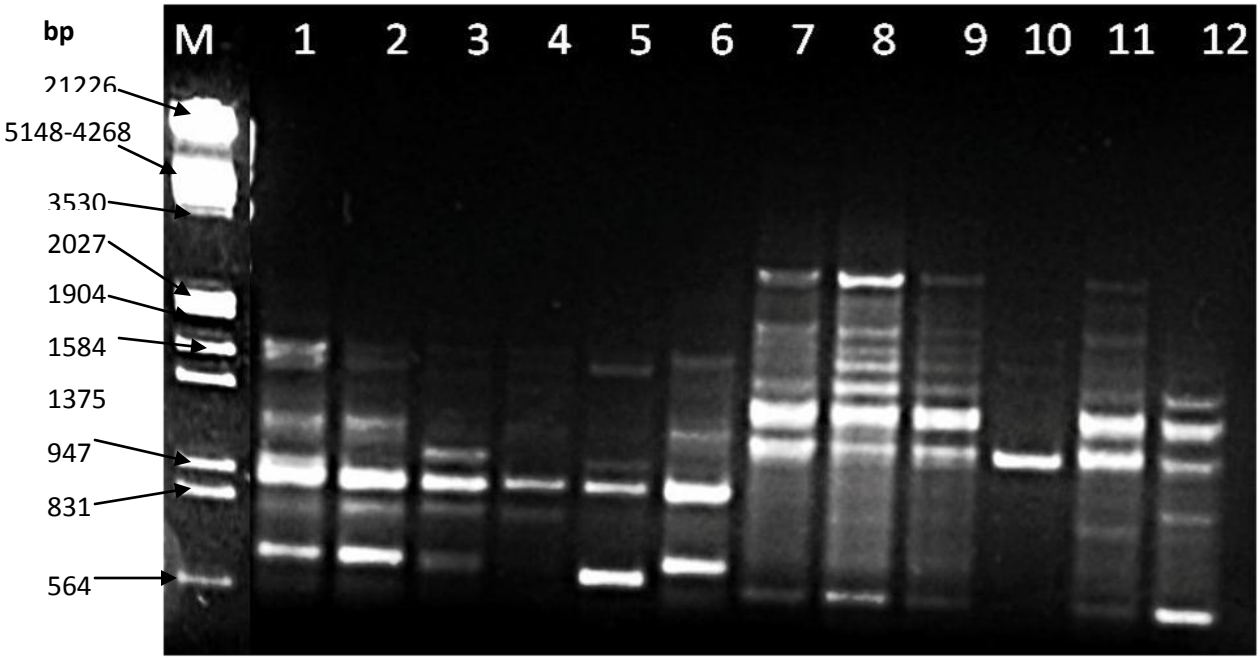

Figure 4.

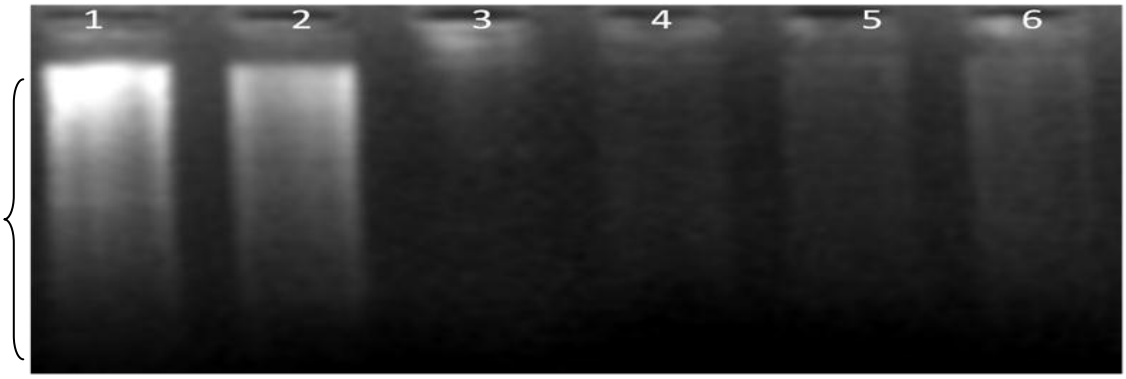

Figure 5.

Figure 1. Electrophoretic pattern of genomic DNA isolated using 1st modification.

Lane M-Molecular weight marker λ DNA EcoRI/Hind lll double digested, Lane1-with liquid nitrogen, Lane 2-alcohol (60%), Lane 3-alcohol (70%), Lane 4-alcohol (80%), Lane 5-alcohol:chloroform (60:40), Lane 6-alcohol:chloroform (70:30), Lane 7-alcohol:chloroform (80:20), Lane 8-alcohol:EDTA (60:40), Lane 9- alcohol:EDTA (70:30) and Lane 10- alcohol:EDTA (80:20).

Figure 2. Electrophoretic pattern of genomic DNA isolated using 2nd modification.

Lane M-Molecular weight marker λ DNA EcoRI/ HindIII double digested, Lane1-with liquid nitrogen,

Lane 2-alcohol (60%), Lane 3-alcohol (70%), Lane 4-alcohol (80%), Lane 5-alcohol:chloroform (60:40), Lane 6-alcohol:chloroform (70:30), Lane 7-alcohol:chloroform (80:20), Lane 8-alcohol:EDTA (60:40), Lane 9- alcohol:EDTA (70:30) and Lane 10- alcohol:EDTA (80:20).

Figure 3. Electrophoretic pattern of genomic DNA isolated using 3rd modification.

Lane M-Molecular weight marker λ DNA EcoRI/ HindIII double digested, Lane1-with liquid nitrogen, Lane 2- alcohol (60%), Lane 3-alcohol (70%), Lane 4-alcohol (80%), Lane 5-alcohol:chloroform (60:40), Lane 6-alcohol:chloroform (70:30), Lane 7-alcohol:chloroform (80:20), Lane 8-alcohol:EDTA (60:40), Lane 9- alcohol:EDTA (70:30) and Lane 10- alcohol:EDTA (80:20).

Figure 4. Electrophoretic pattern of RAPD products generated with primers RP-1 (lane 1-6) and RP-2 (lane 7-12) for different modifications (1d, 2b and 3a).

Lane M-Molecular weight marker λ DNA EcoRI/ Hind III double digested, Lane 1: 5% CTAB (with liquid nitrogen), Lane 2: 5% CTAB (without liquid nitrogen), Lane 3: CTAB+ SDS (with liquid nitrogen), Lane 4: CTAB+ SDS (without liquid nitrogen), Lane 5: 2% SDS (with liquid nitrogen), Lane 6: 2% SDS (without liquid nitrogen), Lane 7: 5% CTAB (with liquid nitrogen), Lane 8: 5% CTAB (without liquid nitrogen), Lane 9: CTAB+ SDS (with liquid nitrogen), Lane 10: CTAB+ SDS (without liquid nitrogen), Lane 11: 2% SDS (with liquid nitrogen), Lane 12: 2% SDS (without liquid nitrogen).

Figure 5. Restriction digestion pattern of DNA extracted by different modifications.

Lane 1 with liquid nitrogen & 2 without liquid nitrogen by 1st modification (1d) , Lane 2 with liquid nitrogen & 3 without liquid nitrogen by 2nd modification (2b), Lane 5 with liquid nitrogen & 6 without liquid nitrogen by 3rd modification (3a).

Table 1. Different primers used for RAPD analysis in *Sesamum indicum* L.

Sr. No.	Primer code	Primer sequence 5'-3'
1	RP 1	CAGGCCCTTC
2	RP-2	TGCCGAGCTG
3	RP-3	AGTCAGCCAC
4	RP-4	AATCGGGCTG
5	RP-5	AGGGGTCTTG
6	RP-6	GGTCCCTGAC
7	RP-7	GAAACGGGTG
8	RP-8	GTGACGTAGG
9	RP-9	GGGTAACGCC

Table 2: Optimization of DNA extraction in *Sesamum indicum* L. using various modifications.

Different extraction buffer				$\lambda_{260}/\lambda_{280}$				DNA concentration (ng/µl)				Yield (µg/g tissue)			
1) 1st modification				A	B	C	D	A	B	C	D	A	B	C	D
	CTAB	PVP	β- m												
1a	2%	1%	1%	1.21	0.89	0.75	0.43	670	400	300	150	1340	800	600	300
1b	2%	4%	2%	1.43	0.49	0.79	0.28	400	230	115	65	800	460	230	130
1c	5%	1%	1%	0.53	0.92	0.98	0.90	95	360	310	275	190	720	620	550
1d	5%	4%	2%	1.74	0.98	1.02	1.25	940	495	575	1045	1880	990	1150	2090
2) 2nd modification															
	SDS	PVP	β- m												
2a	2%	1%	1%	1.21	1.19	1.00	1.10	510	605	405	450	1020	1210	810	900
2b	2%	4%	2%	1.09	0.94	1.20	1.36	855	250	30	320	1710	500	60	640
3) 3rd modification															
	CTAB	SDS	PVP	β- m											
3a	2%	20%	4%	2%	1.08	1.26	1.15	0.99	1180	1105	1360	690	2360	2210	2720	1380

A-DNA extraction with liquid nitrogen, B-DNA extraction without liquid nitrogen (alcohol: EDTA), C-DNA extraction without liquid nitrogen (alcohol: chloroform) and D-DNA extraction without liquid nitrogen (absolute alcohol)