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Full Length Research Paper

Comparison and optimization for DNA extraction of okra (*Abelmoschus esculentus* L. Moench)

Letebrhan Kidane[1], Marie Kalousova[2] and Hewan Demissie[3]*

[1]Tigray Agricultural Research Institute (TARI), P. O. Box 258, Mekelle, Ethiopia.
[2]Czech University of Life Sciences Prague, Faculty of Tropical AgriSciences, Prague - Suchdol 16500, Czech Republic.
[3]Plant Biotechnology, School of Plant and Horticulture Science, College of Agriculture, Hawasa University, P. O. Box 05, Hawassa, Ethiopia.

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The extraction of high-quality DNA from okra (*Abelmoschus esculentus* L. Moench) is notoriously troublesome due to the high contents of polysaccharides, polyphenols, and different secondary metabolites. We have tested seven extraction buffers on silica dried okra leaves. Here, we describe a simple, rapid and modified procedure for high-quality DNA extraction from okra, which is amenable for downstream analyses. In contrast to Cetyl-trimethyl-ammonium bromide (CTAB) methods, the described procedure is rapid, omits the use of liquid nitrogen, phenol, PVP-10, and chloroform. It also uses inexpensive and less hazardous reagents and requires only ordinary laboratory equipment. The procedure employed a high concentration of Sodium dodecyl sulphate (SDS) to rid the problems associated with polysaccharides and polyphenols. The average yield was between 36 and 45 µg of total DNA from 90 mg of dried leaf weight. The DNA is adequate for molecular analysis of okra, such as genetic mapping or marker-assisted plant breeding. This protocol can be performed in as little as 3 h and may be adapted to high-throughput DNA isolation.

Key words: PVP-10, polyvinylpyrrolidone, non cetyltrimethylammonium bromide, okra, genomic DNA, Sodium dodecyl sulphate (SDS).

INTRODUCTION

Okra (*Abelmoschus esculentus* L. Moench), belonging to the Malvaceae family, is an edible vegetable species that is widely cultivated and distributed in the tropical, subtropical and warm temperate areas of the world (Kumar et al., 2013; Lamont, 1999; Diizyaman, 1997; Martin, 1982). The crop is believed to be originated in Ethiopia (Harlan, 1969). It is usually cultivated for its edible immature fruit in fresh or dried form. Nevertheless, the plant is also used as a source of protein (Gemede et al., 2015), fiber (Khan et al., 2017), biomass (Lee et al., 2018), oil (Wei et al., 2017), mucilage (Fronda et al., 2018), colorants (Waghela and Khan, 2018), traditional medicine (Dubey and Mishra, 2017), pharmaceuticals (Zhang et al., 2018) and as ornamental crop (Diizyaman, 1997).

Okra is a highly diversified crop (Akotkar et al., 2010). The wide economic importance of the plant is urging its improvement for different purposes. Thus, breeding elite varieties and developing different cultivars is needed. Even though okra has a wide range of importance and its

*Corresponding author. E-mail: hewan.dd@gmail.com.

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center of origin and diversity is in Ethiopia (Harlan, 1969), molecular variability studies and cultivar improvement programs are still at their infancy.

For studying the molecular characteristics and genetics of okra, extraction of high-quality DNA in sufficient quantity is important. However, extraction of high quality and quantity DNA is often a limiting factor in okra (Porebski et al., 1997), since a large number of species from the Malvaceae family contain secondary metabolites like mucilage (Singh and Kumar, 2012) including alkaloids, phenolic compounds, gummy polysaccharides, terpenes and quinine (Ali et al., 2019; Amani et al., 2011; Porebski et al., 1997; Singh and Kumar, 2012).

During cell lysis, nucleic acids come into contact with these polysaccharides in the oxidized form. The polyphenols bind covalently and irreversibly to proteins and nucleic acids resulting in a brown gelatinous material (Agrawal et al., 2016). This reduces the yield and purity of DNA. Thus, the quality and quantity of DNA will interfere with the subsequent reactions such as PCR, gene cloning and restricted DNA digestion, and sequencing.

Several protocols have been developed for genomic DNA extraction for okra (Jeyaseelan et al., 2019; Seth et al., 2018; Singh and Kumar, 2012). However, these protocols involve the use of hazardous and technologically demanding chemicals like phenol, chloroform, and liquid nitrogen. These chemicals are not recommended in open laboratories with no specialized rooms and safety cabinets. For these reasons, an effective and appropriate protocol is needed for isolating genomic DNA for genotyping, PCR work and sequencing of okra with inexpensive resources while avoiding the health implications of phenol and chloroform (Mahuku, 2004).

The objective of this study was to develop a straightforward technique to isolate deoxyribonucleic acid, a way that eliminates the necessity to use phenol or chloroform to purify the DNA. The resulting SDS (Sodium dodecyl sulphate) protocol was used to isolate high quality genomic DNA subject to PCR analysis from different accession of okra with reduced cost and health concerns. The results will be used for further molecular studies. It also supports technology inaccessible countries to exclude the high cost and impact of phenol-chloroform, liquid nitrogen and enzymes like RNase.

MATERIALS AND METHODS

The DNA extraction process was carried out at the Plant Cell Laboratory, School of Plant and Horticultural Sciences, Hawassa University. Young and healthy leaves from forty-four okra accessions grown in the field were collected from the agricultural field of Hawassa University. The leaves were divided into two batches, one batch was dried with silica gel and the fresh samples were kept in plant collection bag for temporary use.

Solutions

Seven genomic DNA extraction buffers (KCL, CTAB, and SDS) were used with and without modifications in the buffer composition (Table 1).

Protocol for SDS based DNA extraction

1. Ninety (90) mg of silica dried okra leaf tissue were ground to a fine powder with the help of three metal tungsten carbide beads over the vortex mixer supported by micropipette tip based crushing of a sample in an Eppendorf tube.
2. Eight hundred (800) µl of cell lysis buffer (0.5% SDS (w/v) in 10XTE) was added to each tube followed by vortexing at high speed for approximately 2 min until the powder was fully hydrated and mixed with buffer.
3. Samples were incubated for 10 min at room temperature (RT).
4. This step was followed by precipitation of genomic DNA with 200 µl 3 M sodium acetate (pH 5.2) and mixed by inversion of tubes.
5. The mixture was incubated on ice for 5 min.
6. Samples were centrifuged at 16,000Xg for 5 min at RT to pellet the leaf material.
7. The liquid material was transferred carefully to an empty 1.5 ml centrifuge tube.
8. An equal volume of isopropanol was added to the supernatant and completely suspended by vortexing and inverting the tubes (approximately 20 s).
9. Samples were incubated for 15 min at RT by inverting tubes every three minutes by hand.
10. The samples were centrifuged at 16,000Xg for 3 min at RT followed by removal of supernatant with a pipette.
11. 500 µl of freshly prepared wash buffer (5 M NaCl and 95% ethanol) was added to each tube and completely suspended by vortexing the tubes (approximately 20 s).
12. The step was followed by centrifuging the sample at 16,000Xg for 3 min at RT to pellet the genomic DNA.
13. The last step was the removal of the supernatant and washing the pellet with 75% cold ethanol (4°C).
14. The pellet was allowed to dry at room temperature before elution with 60 µl of 1X TE buffer.
15. The DNA was stored temporarily at 4°C before checking its quality and quantity.

Protocol for KCl based DNA extraction

1. Ninety (90) mg of silica dried okra leaf tissue was weighted.
2. Sample was placed into a 1.5 ml tube.
3. 400 ul of DNA extraction buffer (1 M KCl, 100 mM Tris-HCl, 10 mM EDTA) was added to the tube.
4. Sample was crushed by the tip of the pipette inside the Eppendorf tube.
5. Sample was incubated for 30 min (~1 h) at 65°C in a water bath and consequently centrifuged for 10 min at 15000 rpm at 25°C.
6. 100 µl of isopropanol was added to a new 1.5 ml tube or 96-well plate while waiting.
7. 100 µl of supernatant was transferred into the tube prepared in step 6.
8. Samples were mixed by pipetting or inverting tube.
9. The mixture was centrifuged at maximum speed (>2800 rpm) for 30 min at 4°C.
10. The supernatant was discarded by inverting the tube.
11. 150 µl of 70% ethanol was added to wash the pellet.
12. The mixture was centrifuged at maximum speed (>2800 rpm) for 15 min at 4°C.
13. The supernatant was discarded by inverting the tube.
14. The tube was placed upside down for about 10 (~30) min to dry.
15. 30 µl of 1xTE were added to elute the DNA.
16. Samples were kept at 4°C for at least one day to elute well.

Table 1. Composition and concentration of chemicals used for DNA extraction buffers.

Chemical	Lysis Buffer (LB)						
	LB1	LB2	LB3	LB4	LB5	LB6	LB7
NaCl (M)	-	1.4	1.4	-	-	1.4	-
KCl (M)	1	-	-	-	1	-	-
EDTA (mM)	10	20	20	-	10	20	-
Tris-HCl (mM)	100	100	100	-	100	100	-
CTAB (%)	-	2	2	-	-	2%	-
PVP (%)	-	1%	-	-	-	-	-
SDS (%)	-	-	-	0.5	2	-	0.5
βMercapto ethanol (µl)	-	-	2	-	-	-	-
Extraction method	KCL	CTAB	CTAB	SDS	KCL	CTAB	SDS
Modifications	-	1% PVP	2%ß mercapto ethanol	-	2% SDS	1% PVP	Isopropanol precipitation
Reference	Unpublished	Allen et al. (2006)	Allen et al. (2006)	Xia et al. (2019)	-	Unpublished	Xia et al. (2019)

Protocol for CTAB based DNA extraction

Protocol for CTAB was followed according to Devi et al. (2013).

Gel electrophoresis

2 µl of eluted DNA were subjected to electrophoresis on a 0.8 mg/ml ethidium bromide stained 1% (w/v) agarose (Thermo Scientific, USA) gel in 1XTris-EDTA (TE) buffer. The agarose gel was documented on a GelDoc (BIO-RAD, USA).

Qualitative and quantitative analyses of the isolated DNA

The yield and purity of extracted DNA was assessed using Nano-Drop 2000 (Thermo-Scientific, USA), by measuring the concentration and UV absorption ratios at A260/280 and A260/230. Agarose gel electrophoresis was performed to determine the level of DNA degradation and contamination by RNA.

PCR amplification confirmation

PCR was carried out in a thermal cycler (BIO-RAD, USA) to amplify the specific DNA sequence, in a reaction volume of 10 µl containing PCR buffer (10 mMTris-HCl, 50 mMKCl), 50 ng of the DNA, 1.5 mM $MgCl_2$, 0.5 units of Taq polymerase (Fermentas, USA), 0.1 mM of dNTPs (Fermentas, USA), 10 pM of each ribosomal gene-specific 18S forward (5`-AACGGCTACCACATCCAAGG-3`) and reverse (5`-TCATTACTCCGATCCCGAAG-3`) primers. The amplification conditions were: initial denaturation for 5 min at 95°C, followed by 30 cycles of 45 s denaturation at 94°C, 45 s annealing at 55°C and 45 s extensions at 72°C. Final extension step was at 72°C for 10 min.

Two SSR primers (ST-1) synthesized by (Metabion international AG, Germany) were used for detecting the functionality of the extracted DNA by this developed protocol. The PCR reaction (10 µl volume) contained about 50 ng of genomic DNA, 0.1 µl of blend taq polymerase (2.5 u/µl, Top-Bio s.r.o., Czech Republic), 2 µl 10× PCR reaction buffer, 0.1 µl $MgCl_2$ (25 mM), 0.2 µl dNTP mixture (2.0 mM), 0.2 µl each primer (25 µM), and dH_2O up to 10 µl. Amplification was carried out in a T100 BIO-RAD thermal cycler. Cycling conditions consisted of 5 min initial denaturation at 95°C, followed by 1 min denaturing at 95°C, 1 min annealing at 55°C and 1 min extension at 72°C repeated for 35 cycles and 5 min extension at 72°C. PCR products were subsequently separated by electrophoresis on 2% agarose gel at 70 V for 40 min, which was then stained with ethidium bromide (0.5 mg/ml) and photographed as described above by using 1.5 µl of 1 kb DNA ladder.

Data analysis

Data were analyzed on the DNA concentration and on the 260/280 and 260/230 ratios by agricolae package (de Mendiburu, 2020) using the R software version 3.5 (R Core Team, 2018). Tukey's pairwise comparisons with the confidence interval of 95% were used to compare the concentration between the extraction methods.

RESULTS AND DISCUSSION

This method largely follows those already developed for other plant species in the major steps, such as cell disruption, DNA extraction and precipitation (Kalbande et al., 2016; Paterson et al., 1993); however, there were modifications to the composition to the lysis buffer, which were intended to overcome the issues of high secondary metabolite content in the plant tissue. Specifically, a phenol-binding reagent (SDS) and NaCl were used to remove polyphenols and polysaccharides, respectively.

In addition, to reduce the cost and processing time of the procedure, all buffers were prepared from chemicals available in local stores.

The analyzed results for the ratio of UV absorption $A_{260}/_{280}$ and $A_{260}/_{230}$ and the concentration of DNA extracted by the different modified KCl, CTAB and SDS methods are presented in a box plot (Figures 1 and 2). The mean concentration and quality of DNA is presented in Table 2. Comparison of quality and quantity values (Table 2) indicated that there is no significant difference between different accessions on: the concentration ($F = 0.34$, $df = 3$, $P = 0.08$), $A_{260/280}$ ratio ($F = 0.26$, $df = 3$, $P = 0.86$), and the $A_{260/230}$ ratio ($F = 0.18$, $df = 3$, $P = 0.91$). This contradicts with the principle that plant species belonging to the same or related genera can exhibit

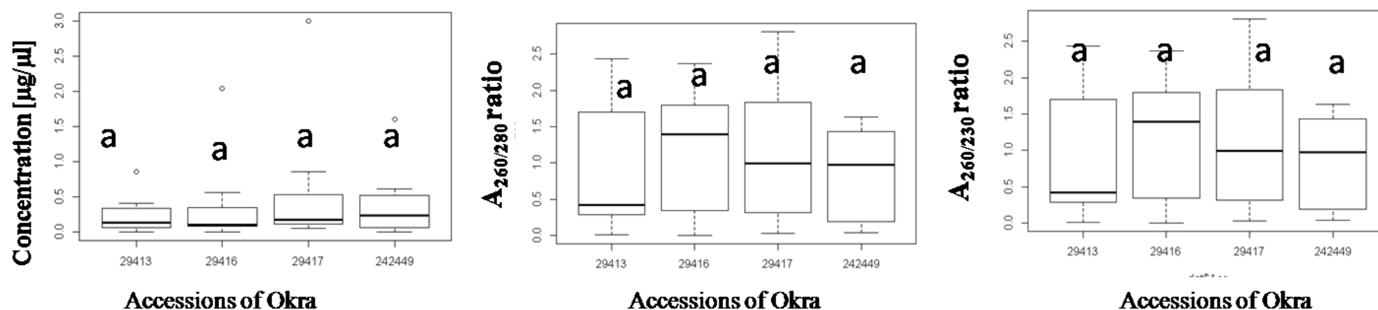

Figure 1. Box plot of DNA concentration and OD value from four Accessions of Okra using the seven different extraction buffers.

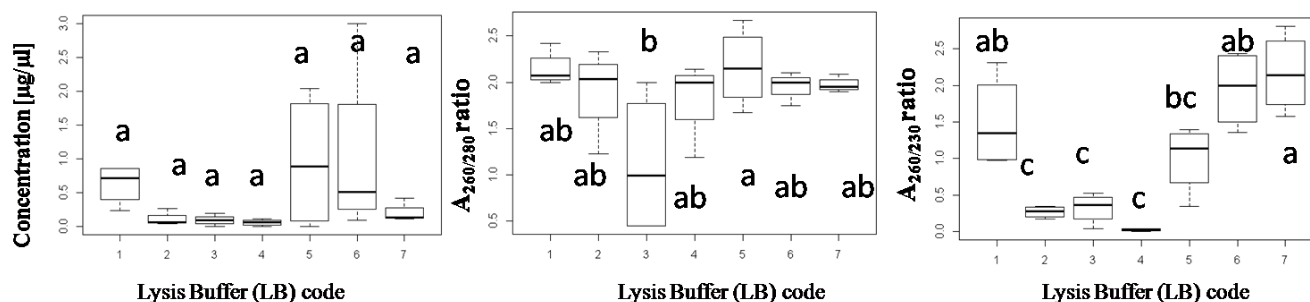

Figure 2. Box plot of DNA concentration and OD value of okra DNA extracted using the seven different extraction buffers.

Table 2. Analysis of variance on the concentration, 260/280 and 260/230 ratios of the seven extraction buffers used in the experiment.

Parameter	Lysis buffer			Accessions		
	Concentration (µg/µl)	$A_{260/280}$ ratio	$A_{260/230}$ ratio	Concentration (µg/µl)	$A_{260/280}$ ratio	$A_{260/230}$ ratio
DF	6	6	6	3	3	3
Sum of square	4.34	3.03	17.87	0.53	0.21	0.49
Mean square	0.72	0.51	2.98	0.18	0.07	0.16
CV	148.68	22.82	41.24	166.35	28.14	91.04
MSD	1.58	1.04	1.04	1.12	0.81	1.44
F value	1.72	2.78	16.44	0.34	0.26	0.18
Pr (>F)	0.17	0.04	0	0.8	0.86	0.91
Significance	NS	*	***	NS	NS	NS

NS – not significant, * - significant at $P \leq 0.05$, *** - significant at $P \leq 0.001$.

enormous variability in their biochemical composition and this heterogeneity may not permit optimal DNA yields from one isolation protocol and this leads to the development of different isolation protocol even for closely related genera. However, significantly different results were obtained comparing the different lysis buffers (Table 2) for $A_{260}/_{280}$ratio ($F = 2.78$, df = 6, $P = 0.04$) and for $A_{260}/_{230}$ratio ($F = 16.44$, df = 6, $P = 0.00$). Intactness and quality difference of the DNA extracts using the different lysis buffers is shown in Figure 3. The assessment of the purity of the DNA is confirmed by the $A_{260}/_{280}$ ratio. For a 'pure' nucleic acid, this value commonly resides in the range of 1.8 to 2.0 (Sambrook et al., 1989). The $A_{260}/_{280}$ ratios below approximately 1.3 and above 2.3 are indicators of poor quality of the DNA (Seth et al., 2018). Samples with absorbance ratio at $A_{260}/_{280}$ greater than 2 indicate the presence of carbohydrates and other secondary metabolites (Wilson and Walker, 2010). Higher values of absorbance ratios are evidence of contamination by phenols while lower values indicate the presence of proteins since proteins absorb light at a wavelength of 280 nm (Wilson and Walker, 2010). The presence of RNA in the sample has been also shown to increase the $A_{260}/_{280}$ ratio. RNA contamination can be

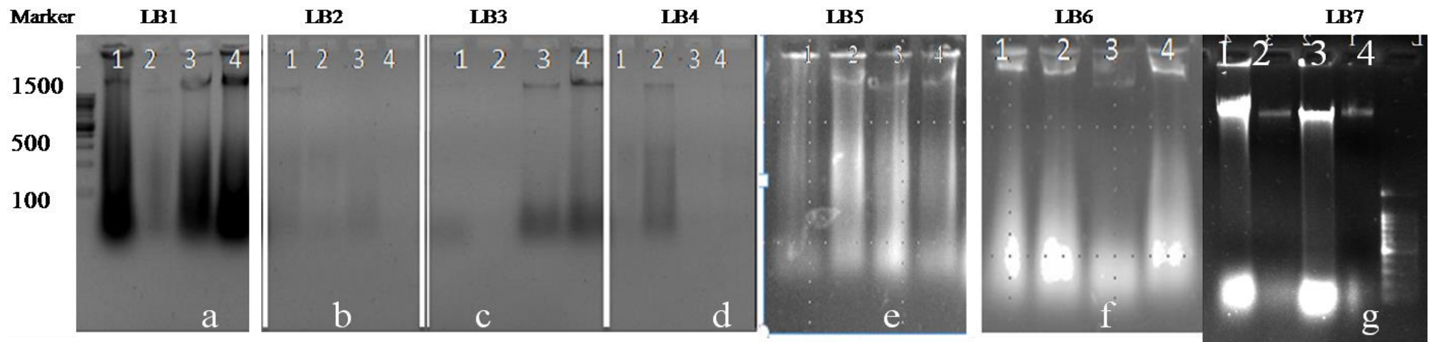

Figure 3. Electrophoresis of Okra DNA on 1% agarose gel. From a to g is the different extraction buffer used as indicated in Table 1. Lanes 1 to 4 indicates the different Okra accessions used for the experiment (1=29416; 2= 29417; 3= 242449; 4= 29413). The first and last lane is the 1.5 Kbp and 100 bp DNA ladder marker respectively.

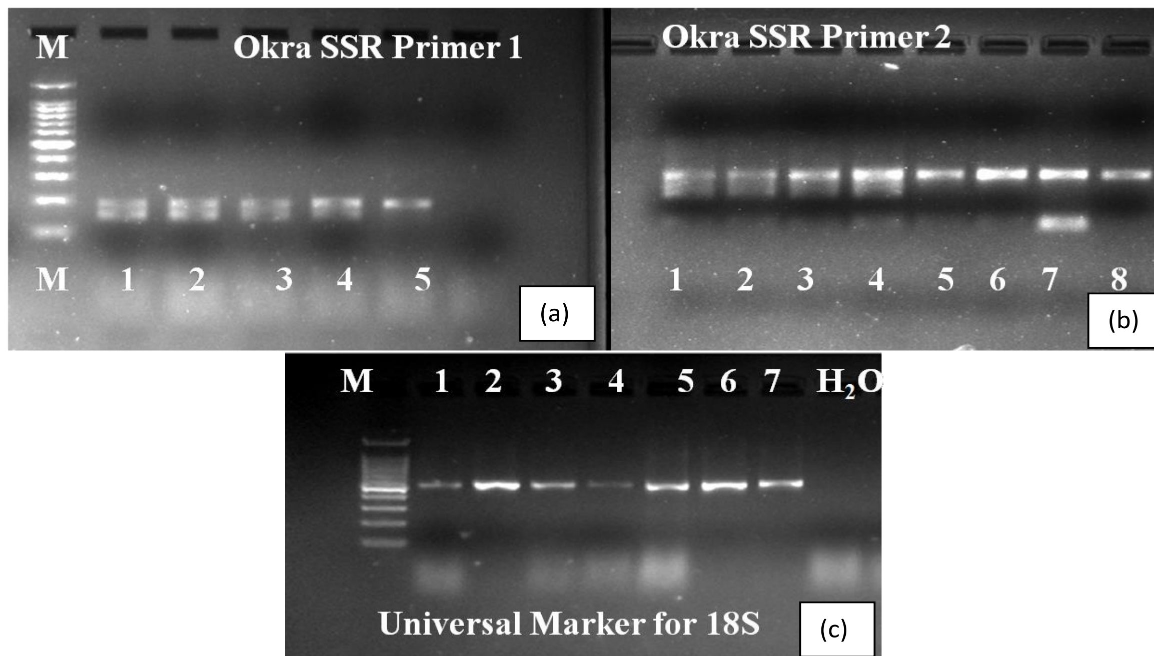

Figure 4. Two SSR and one Universal (18S) Primer patterns were used for the amplification of extracted Okra DNA using LB7 of representative Okra genotypes (a) Okra SSR marker 1 (b) Okra SSR marker 2 (c) Universal marker (18S) for the different samples of Okra (Lane 1 to to 8. M: 100 bp ladder).

confirmed or ruled out by agarose electrophoresis, in case of RNA being present in the sample; it has to be treated by RNase (Valledor et al., 2009).

In this experiment, the majority of the lysis buffers resulted in the formation of smear on the gel, which indicated the degradation of the DNA (Figure 3). However, lysis buffer 7 (LB7) formed a relatively better band with less smear, which also hints at absence of RNA contamination (Figure 3). The absorbance ratio of $A_{260/280}$ for the four tested samples ranged between 1.9 and 2.0. The values of all samples were within the accepted range, indicating a low level of contamination (ST-2). Similar work was also conducted for extracting DNA from samples with a high amount of polysaccharides and mucilage using SDS to replace CTAB extraction buffer (Sharma et al., 2018). SDS is an anionic detergent for cell and nucleus lysis to release ribonucleic and deoxyribonucleic acids by inhibiting the nucleases, ribonuclease (RNase) and deoxyribonuclease (DNase) activities (Farrell, 2011). The resulting DNA was further used for SSR marker-based analysis (Figure 4).

CTAB based DNA extraction method is the most commonly used technique for different crop species. However, in our current experiments CTAB was not satisfactory in terms of quality and quantity of DNA with/without modification and with modification of CTAB

extraction components like PVP, β- mercaptoethanol (Figure 3). The extraction of DNA from fresh okra samples using CTAB resulted in a thick and sticky substance, which could not be pipetted out of the Eppendorf tubes. This can be explained by the presence of polysaccharides in the DNA sample which form a highly viscous solution (Jeyaseelan et al., 2019). Mucilage is a highly viscous secondary metabolite composed of a polar polymer of glycoprotein that can co-precipitate with DNA and inhibits the action of Taq polymerase (Menu et al., 2018). Polysaccharides are problematic as they make the DNA unreliable during pipetting and hinder the activity of polymerases and restriction endonucleases (Kumar et al., 2018).

Conclusion

This study compared and optimized quality and speedy protocol which will be used for routine DNA isolation from okra (*A. esculentus* L. Moench) and is amenable for marker-assisted breeding, and high-throughput applications. In addition, this protocol may be used for other plant species that are recalcitrant to other methods due to their high levels of polysaccharides and polyphenols.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary Tables (ST)

Table S1. List of primers used for PCR amplification of the genomic DNA extracted by the improved protocol.

Primer	Primer Sequence	Type	Expected Size	PIC	TM (°C)
18S-FW	AACGGCTACCACATCCAAGG	Universal	500	-	55
18S-RW	TCATTACTCCGATCCCGAAG	Universal			55
Ok1-FW	TCATGTCTTTCCACTCAACA	SSR	194	0.58	54
Ok1-RW	CCAAACAAAATATGCCTCTC	SSR			54
Ok3-FW	AACACATCCTCATCCTCATC	SSR	203	0.73	56
Ok3-RW	ACCGGAAGCTATTTACATGA	SSR			54

Table S2. DNA yields, 260/280 and 260/230 ratios obtained from (Lysis buffer 7 (LB-7) for the 44 Okra genotypes.

Accession	Concentration (µg/µl)	$A_{260/280}$ Ratio	$A_{260/230}$ Ratio
24213	0.23	1.8	0.9
29408	0.77	2	1.2
29409	0.45	1.9	1
29410	0.75	1.9	0.9
29411	0.53	1.7	0.8
29412	1.13	2	1.4
29413	0.57	2	1.1
29414	2.22	2.1	1.5
29415	1.09	2	1.3
29416	1.18	2.1	1.5
29417	0.88	1.8	0.9
240201	2.13	2.2	1.7
92203	0.9	2	1.5
240203	0.64	2	1.4
240204	0.59	2	1.3
240207	0.91	2.1	1.5
240209	1.1	2.1	1.5
240583	1.27	2.2	1.7
240585	1.35	2.2	1.9
240586	1.31	2.1	1.7
240587	0.31	2.1	2.1
240591	0.34	2.1	1
240592	0.34	2	1.4
240599	0.32	1.8	1.3
240600	0.43	1.8	0.8
240601	0.59	2.1	1.6
240602	0.32	1.9	1.1
240609	0.53	1.9	1.4
240615	0.68	2	1.6
240784	0.54	2.1	1.9
240786	1.94	2.1	1.7
242433	0.34	2.1	1.8
242443	0.71	2.2	1.8
242444	0.57	2.1	1.5
242445	0.56	2.1	1.5
242448	0.58	2.2	2
242449	0.6	2.2	1.9

Table S2. Contd.

New	**0.5**	**2.1**	**1.6**
242450	0.26	2	1.6
242451	0.52	2	1.5
245161	0.38	1.9	1
245162	0.47	2.1	1.4
Local	0.47	2	1.3
New	0.98	2.2	2
Improved	0.47	2.1	1.5