IMPROVED METHODS FOR VIRAL DNA EXTRACTION FROM OKRA (*Abelmoschus esculentus* L. Moench) - OVERVIEW

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Abstract. *Molecular techniques require isolation of genomic DNA of suitable purity. High-quality genomic DNA is one of the major requirements for sharp band amplicons in a polymerase chain reaction for molecular detection of viruses on okra. The presence of mucilaginous acidic polysaccharides, polyphenols, and different secondary metabolites in okra leaves interfere with this process to a large extent as it makes DNA unmanageable in pipetting and inhibit Taq DNA polymerase activity. In this study, we reviewed improved and reliable extraction protocols which are efficient for highly purity DNA isolation from okra leaves, flowers and seeds. Several procedures have been adopted in recent years such as the modifications of conventional CTAB; increase the volume of DNA extracting buffer (1.5ml/sample), decrease sample volume (50-60 mg), higher salt concentration (5M NaCl), use of polyvinylpolypyrrolidone, omission of chloroform. Other procedures employed high concentration of Sodium dodecyl sulphate (SDS) and Potassium Chloride (KCl). The yield and quality of the isolated DNA was free of contaminants, suitable for further genomic analysis through PCR, RAPD, and high-throughput DNA isolation. These modified methods are very easy to conduct, do not necessarily require liquid nitrogen, use of cost-effective laboratory materials and can be carried out within a short period of time.*

Keywords: *Molecular technique, DNA extraction, Okra, mucilage, CTAB, SDS*.
Abbreviation: *CTAB-Cetyltrimethylammonium bromide; PVP-polyvinylpyrrolidone, PCR-Polymerase Chain Reaction, NaCl- Sodium chloride*.

INTRODUCTION

Okra (*Abelmoschus esculentus* Moench) is an edible vegetable widely cultivated and utilized among members of the family *Malvaceae* (NAVEED *et al.*, 2009). It is called lady's finger in England, *gumbo* in USA, *bhendi* in India, *Kenkase* in Ethiopia, *kubewa* in Nigeria and *bame* in Romania (SORAPONG, 2012). It is a widely cultivated economic crop produced in small or commercial scale within tropical, subtropical and warm temperate regions around the world (SINGH *et al*., 2012). In 2021, world production of okra was over 10.8 million tonnes. India ranks first with a production of 6.47 million tonnes (about 60%), Nigeria ranks second in world production and highest in Africa with 1.92 million tonnes (about 18%) and Mali ranks third with 0.67 million tonnes (FAOSTAT, 2021).

Okra fruit is principally consumed fresh or cooked and is a major source of vitamins A, B, C, minerals, Iron, Iodine and source of viscous fiber. Okra seeds are source of oil and protein and have also been used on a small scale for oil production, caffeine-free substitute for coffee and confectionary (CALISIR & YILDIZ, 2005). Its medicinal value has been reported in curing ulcers, genito-urinary disorders, spermatorrhoaea, chronic dysentery and relief from hemorrhoids. Studies show that daily consumption of 100 g of okra provides 20% of calcium, 15% iron and 50% of vitamin C of human dietary requirements (ADETUYI *et al.*, 2011).

In this plant, DNA extraction is difficult due to the large amount of mucilage in the leaves, flowers, and pods. Okra mucilage is a viscous slimy glue - like substance found in fresh and dried leaves, flowers and pods. They are chemically acidic polysaccharides associated with proteins and minerals (AHMED *et al.*, 2013). Although the nature of the polysaccharides varies greatly,

neutral sugars rhamnose, galactose and galacturonic acid have been reported as a major problem during the purification of okra DNA (HIROSE *et al.*, 2004; SENGKHAMPARN *et al.*, 2009). They make DNA unmanageable in pipetting and also difficult to separate from isolated DNA and these contaminants inhibit *Taq* DNA polymerase activity and ligases. When cells are lyzed, nucleic acids come into contact with these polysaccharides (LOOMIS, 1974). In the oxidized form these polyphenols bind covalently and irreversibly to proteins and nucleic acids (Guilletmaut and Marechal-Drouard 1992) resulting in a brown gelatinous material that reduces the yield and purity of the extracted DNA (POREBSKI *et al.*, 1997; ALJANABI *et al.*, 1999) and prevents it from redissolving completely. Furthermore, DNA which dissolves even in the presence of these polysaccharides inhibits the activity of different restriction enzymes (SAHU *et al.*, 2012), PCR, or in vitro labelling.

Molecular technique requires isolation of genomic DNA of suitable purity for accurate okra plant disease detection and management. The availability of high quality intact genomic DNA is a precondition for almost every molecular genetic analysis of crops. But when a plant tissue is rich in polysaccharide contaminants, isolation of good quality DNA for PCR, gene mapping, diversity assessments and other molecular analyses is challenging, due to secondary metabolites and polyphenols

Although several protocols for the isolation of DNA from tissues with high levels of polysaccharides and polyphenols have been reported WANG *et al.* (2011). Okra is undoubtedly one of the most difficult plants to work with, following published protocols used by researchers and failing to obtain high quality DNA that was not contaminated with polyphenolic compounds and did not give satisfactory results, also KUMAR *et al.* (2018), were uniformly unsuccessful in their attempts to amplify okra DNA by PCR using other reported methods, including those of DELLAPORTA *et al.* (1984) and COEN *et al.* (1990). Hence, the need for this review. The aim of this study was to harness different conventional and optimized reliable protocols that will ensure DNA isolation with good spectral qualities suitable for RAPD and PCR analysis.

The methods proposed and most often used by researchers are presented in Table 1 and Figure 1.

Table 1

CTAB and Non CTAB-based DNA extraction methods and composition for Okra plant

Protocol	CTAB DNA Extraction	Modified CTAB	More Modified CTAB	SDS	KCl
Sample Size	500 mg-1.0 gm	50-60 mg	100 mg	90 mg	90 mg
Sample ground with	Mortar and pestle	Liquid nitrogen	Not specified	Metal Tungsten carbide beads	Pipette tip
Extraction buffer	2% CTAB (w/v), 1.4 M, NaCl, 0.02 M EDTA, 0.1 M Tris HCl (pH 8.0)	2% CTAB (w/v), 5.0 M NaCl, 0.5M EDTA, 1 M tris-HCl (pH 8.0)	3% CTAB (w/v), 5.0 M NaCl, 0.5M EDTA, 1 M tris-HCl (pH 8.0)	0.5% SDS (w/v), 10XTE	1 M KCl, 100 mM Tris-HCl, 10 mM EDTA)
Volume/ sample	Not stated	1.5 ml	1.5 ml	800 µl	400 µl
Polyvinylpolypyrrolidone:	Not used	Used	3%	Not used	Not used
Beta-mercapto ethanol	Not mentioned	2%	3%	Not used	Not used
Incubation temperature/time	60°C for 30mins	65°C for 60 mins	65°C for 60 mins	10 min at room temperature	65°C for 30 mins
Isopropanol	0.6 volume	0.7 volume	Not used	Equal volume	Equal volume
Washing	500 µl 76% ethanol	1 ml 70% ethanol, absolute ethanol	95% ethanol, 70%	500 µl (5 M NaCl and 95% ethanol, 70%)	150 µl 75% cold ethanol
R-Nase	Not used	1.5 µl	Not stated	Not used	Not used
Reference	Doyle and Doyle (1987)	Singh *et al.*, (2012)	(Unpublished)	Kidane *et al.*, (2020)	Kidane *et al.*, (2020)

MATERIAL AND METHODS

This paper is a review of the methods currently used for DNA extraction. To carry out this work, classical documentation methods were used, such as: simple text analysis techniques,

bibliometrics, semi-systematic. The scientific papers analyzed for the topic of this review were subsequently compared to highlight the best methods for DNA extraction.

In this study, we reviewed improved and reliable extraction protocols which are efficient for highly purity DNA isolation from okra leaves, flowers and seeds.

Reliable and optimized methodologies for DNA extraction from okra

The extraction of DNA from okra plant material starts with the sample collection from the leaves, flowers or seeds of okra. The collected sample if fresh in the case of leaves and flowers need to be stored under clean and hygienic condition such as the use anhydrous calcium chloride, refrigerated or air dried to avoid secondary infection or discoloration of the sample.

Broadly the different methods of extracting genomic DNA from plants can be classified into two (2) namely:

i. CTAB based

ii. Non-CTAB based (Tabel 1; Figure 1).

Doyle and Doyle modified CTAB

Take 50 - 60 mg of fresh leaves of okra and grinded with liquid nitrogen, after thawing ground the sample in mortar with a preheated CTAB extraction buffer (1.5 ml/sample) with 0.2% 2-betamercaptoehanol (Table 1). Incubate at 65^{o}C for 60 mins, after that add 1.5 µl of RNase and incubate at 37^{0}C for 20 mins. Centrifuge at 12,000 rpm for 10 mins to pellet the debris then add equal volume of Chloroform: Isoamyl alcohol (24:1 v/v) to the supernatant and gently vortex for 10 mins and centrifuge 13,000 rpm for 10 mins. Transfer the supernatant into 0.7 volume of ice-cold Isopropanol and 0.15 volume ammonium acetate to precipitate DNA at -20^{o}C for 30 min. Washing done twice by adding 500 µl of 70% chilled ethanol to remove ions and then from absolute ethanol. Centrifuge at 13000 rpm for 1 min to pellet the DNA, then air dry and re-suspend in 50 µl of TE buffer (Tabel 1; Figure 1).

Sodium dedocyl sulphate -SDS DNA extraction

Three metal tungsten carbide beads over the vortex mixer was used to grind to fine powder 90mg of okra leaf. Then (800) µl of cell lysis buffer (0.5% SDS (w/v) in 10 XTE) 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. Samples were incubated for 10 min at room temperature (RT), followed by precipitation of genomic DNA with 200 µl 3 M sodium acetate (pH 5.2) and mixed by inversion of tubes. The mixture was incubated on ice for 5 mins and centrifuged at 16,000Xg for 5 min at RT to pellet the leaf material. The supernatant was transferred carefully to an empty 1.5 ml centrifuge tube. An equal volume of isopropanol was added to the supernatant and completely suspended by vortexing and inverting the tubes (approximately 20 s). Samples were incubated for 15 min at RT by inverting tubes every three minutes by hand. The samples were centrifuged at 16,000Xg for 3 min at RT followed by removal of supernatant with a pipette, freshly prepared wash buffer 500 µl of (5 M NaCl and 95% ethanol) was added to each tube and completely suspended by vortexing the tubes (approximately 20 s). The step was followed by centrifuging the sample at 16,000 Xg for 3 min at RT to pellet the genomic DNA. Lastly the supernatant was discarded and the pellet was washed with 75% cold ethanol (4°C). The pellet was allowed to dry at room temperature before elution with 60 µl of 1X TE buffer and DNA was stored temporarily at 4°C before checking its quality and quantity (Tabel 1; Figure 1).

Potassium Chloride (KCl) DNA extraction

Ninety (90) mg of silica dried okra leaf tissue was weighted. The sample into a 1.5 ml tube, after which 400 ul of DNA extraction buffer (1 M KCl, 100 mM Tris - HCl, 10 mM EDTA) was added to the tube and was crushed by the tip of the pipette inside the eppendorf tube. Sample was incubated for 30 - 60mins at 65°C in a water bath and consequently centrifuged for 10 min at 15,000 rpm at 25°C. Then, add equal volume of isopropanol to the supernatant and mixed by inverting tube. The mixture was centrifuged at maximum speed (>2800 rpm) for 30 min at 4°C. The supernatant was discarded by inverting the tube followed by the addition of 150 µl of 70% ethanol to wash the pellet and centrifuged at maximum speed (>2800 rpm) for 15 mins at 4°C. Discarded supernatant by inverting the tube for 10-30 mins to dry and finally the DNA was eluted in 30 µl of 1 x TE and keep at 4°C for one day to elute well (Tabel 1; Figure 1).

RESULTS AND DISCUSSION

CTAB based DNA extraction method is the most commonly used technique for different crop species. However, the information presented in the various experiments on okra, CTAB was not satisfactory in terms of quality and quantity of DNA especially when fresh samples are used a thick mucilage was difficult to be pipetted out of the Eppendorf tubes of polysaccharides a secondary metabolite in the DNA sample which form a highly viscous solution (JEYASEELAN *et al.*, 2019).

Modified CTAB and non-CTAB based protocols in this study yielded good quality DNA as compared to the conventional CTAB. Different modification in Doyle and Doyle method helps to remove mucilage and polyphenols in okra leaves for purified DNA suitable for PCR amplification and RAPD analysis. One important modification in given method, use of DNA extraction buffer 1.5 ml/sample is key in all modification. SING (2012) achieved this, by increasing the volume of DNA extraction buffer and decreasing the amount of plant material which help to remove majority of polysaccharides with high salt concentration. Salt use during precipitation of DNA increase solubility of polysaccharides in ethanol thus preventing co-precipitation with DNA, concentration of 5 M NaCl when add to the suspension in the lysis step did result in the highest yields of total genomic DNA. The use of PVP enhanced binding to polyphenols compounds and make separation from DNA by centrifugation better POOJA *et al.* (2012) and KIDANE *et al.* (2020).

The presence of oxidized phenolic compounds can be reduced further by keeping plant material frozen during homogenization. Ahmed *et al.*, 2013 reported that the modified protocol yielded 8 - 10 µg high-quality intact genomic DNA from 100 g okra mature and young leaves applicable to enzymatic digestion and PCR amplification in 1.5 mL tube without using liquid nitrogen.

Furthermore, DNA prepared with this optimized procedure was tested for yield, purity, susceptibility to restriction endonuclease digestion and ability to be amplified by PCR. High-quality DNA 100 mg fresh leaves, enough for 500 RAPD reactions was obtained. The A260/A280 of the extracted DNA ranged from 1.6 - 2.1 consistent with the ratio recommended for pure DNA preparations. The use of high concentration NaCl with isopropanol helps the release of DNA from polysaccharides. The polysaccharides settle in the bottom with NaCl and precipitated DNA remains suspended in the isopropanol in the upper layer was reported by NISAR *et al.*, (2013).

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 (KIDANE *et al.*, 2020). 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 (SHARMA, 2018).

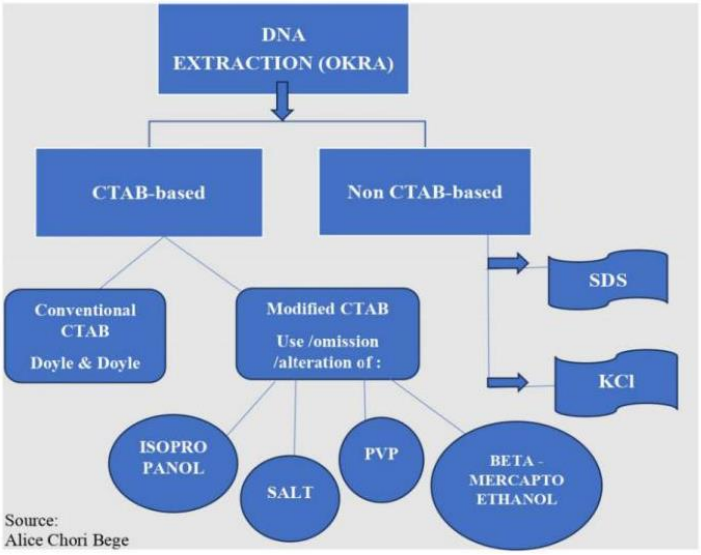

Figure 1. Concept Map

These protocols are easy to carryout, liquid nitrogen is not required and yields hight quality DNA without impurities which is very suitable for PCR and further molecular analysis. Similar conditions on a large scale, using 1-10 g samples, can be scaled up and fit into a 50 ml screw capped tube. The method may also be beneficial for other plant species tissues containing high levels of polysaccharides.

CONCLUSIONS

Okra viruses are very damaging pathogens because they cannot be controlled with chemicals. With the help of chemicals, insect vectors can be controlled. Unfortunately, in Nigeria, different viruses are present in okra every year, both in the rainy and dry seasons, especially in the North - West, where it is grown on large areas.

Following this review, we can conclude that the best DNA extraction methods are the Modified CTAB and More Modified CTAB, as they result in DNA of the highest quality, compared to other methods presented in this paper where the quality is much lower.

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