Selecting and optimizing a reliable DNA extraction method for isolating viral DNA in okra (*Abelmoschus esculentus*)

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Abstract— Detection and identification of viruses in okra plant is important to manage viral diseases. Good quality of DNA is essential for the PCR based detection and subsequent genomic sequencing. However, the DNA extraction has been greatly affected by mucilaginous substances present in okra plant parts. Objective of this study is to find out an extraction method that can eliminate mucilaginous materials and yield good quality DNA. Five different protocols were tested with okra leaf samples having okra yellow vein mosaic disease (OYVMD) symptoms. Quantity and purity of extracted DNA was tested using Nanodrop spectrophotometer. Precise quantity comparison was done by measuring total plant DNA in relation to the copy number of *ACT2* gene using quantitative polymerase chain reaction (qPCR). The extracted DNA samples were used as template for detection of *Bhendi yellow vein mosaic virus* and *Bhendi yellow vein mosaic betasatellite* using specific primers. A modified protocol (modified method 1), introduced in this study, produced better yield and quality of DNA compared to other tested protocols. The method yielded 32 µg DNA for 100 mg leaf powder and the ratios A260/A280 and A260/A230 were 1.8 and 2.1, respectively. The qPCR quantification further confirmed higher quantity of DNA yield in this modified protocol. End point PCR with virus specific primers yielded very bright bands for *Bhendi yellow vein mosaic virus* and *Bhendi yellow vein mosaic betasatellite*. In conclusion, the modified method 1 can be used to extract good quality and quantity of DNA from okra.

Keywords— Okra, DNA extraction, plant viruses

I. INTRODUCTION

Detection and identification of plant viruses is the basis to manage plant diseases and to predict the crop loss by the infection. There are various approaches to detect and identify the viruses associated with diseased plants. Nucleic acid-based diagnostic techniques such as hybridization, amplification of targeted viral genome or genomic component(s) by the polymerase chain reaction (PCR) and DNA sequencing have overwhelmingly replaced the use of microscopical and serological techniques (Jose and Usha, 2000; Sharma *et al.*, 2005; Khan *et al.*, 2007; Kushwaha *et al.*, 2010). Nucleic acid based diagnostic techniques are more reliable, specific and reproducible. Better quality and enough quantity of virus genomic DNA or RNA are pre-requisite since the genomic components of the viruses are mixed with cellular constituents of infected plant tissues (Swanson *et al.*, 1992).

DNA extraction from some plant tissues remains difficult due to the contamination of secondary metabolites (Cavallari *et al.*, 2014). Plants such as Okra (*Abelmoschus esculentus*), Mesta (*Hibiscus cannabinus*), Jute (*Corchorus* sp.) and Sida (*Sida* sp.) possess large amounts of viscous mucilage, which often have secondary metabolites, such as polysaccharides, phenolic compounds, tannins, and alkaloids (Höfer *et al*., 1997; Jose and Usha 2003; Ghosh *et al*., 2008; Roy *et al*., 2009). The viscous mucilage co-precipitates with DNA during total DNA extraction and reduces the quality of the extracted DNA. In addition, attempt to remove the mucilaginous substances always associated with reduction in total DNA quantity (Bayer *et al*., 1999). The mucilage contamination makes the DNA unmanageable during pipetting and interferes with further downstream applications, such as PCR or other enzymatic reactions (Fang *et al.*, 1992). The concentration and viscosity of mucilage varies from one plant species to other or among different cultivars of one species is due to the chemical complexity and variation in the physiological properties (Muralikrishna *et al.*, 1989; Kawamura *et al.*, 2000; Axel Diederichsen *et al.*, 2006)

Finding out a rapid, simple and reliable DNA extraction protocol is most vital in PCR based diagnosis of virus diseases in plants. Cetyl trimethyl ammonium bromide (CTAB) method is one of the most common methods for DNA extraction from plant samples (Doyle and Doyle, 1987). However, a CTAB extraction procedure does not guarantee the elimination of some contaminants such as lipids, phenolic compounds, and viscous polysaccharides (Pedersen *et al.*, 2006). There are several modified versions of CTAB extraction protocols available to extract DNA from virus infected plant tissues (Deng *et al.*, 1994; Mansoor *et al.*, 1999; Rothenstein *et al.*, 2005; Briddon and Markham, 1994).

Jose and Usha (2000) described a protocol for the extraction of geminiviral DNA from infected okra. This protocol is a combination of a modified CTAB method of Dellaporta *et al.* (1983) and a citrate buffer extraction with alkali-lysis. However, the method takes too long to get the extracted DNA.

Ghosh *et al*. (2009) reported that the DNA extracted by standard CTAB method (Doyle and Doyle, 1987) did not respond to PCR or exhibit inconsistent amplification of virus DNA due to the contamination of mucilaginous substances. Even though the isolated DNA was further purified via DNeasy mini spin column (QIAGEN, GmBH, Hilden, Germany) the PCR amplification was not successful. Therefore, Ghosh *et al*. (2009) developed a modified CTAB protocol to extract DNA from highly mucilaginous plant samples. The newly developed method gave enough quantity of DNA to Jute plant. However, this method has few drawbacks, such as utilizing large volume of reagents, time consuming and deals with toxic phenol.

The present study describes an improved, rapid, safe and inexpensive, modified method for DNA isolation which is suitable for further downstream amplification of full or partial genome of begomoviruses and betasatellites. The method described in this study has been standardized with okra plants infected with a begomovirus, *Bhendi yellow vein mosaic virus* (BYVMV) and *Bhendi yellow vein mosaic betasatellite* (BYVMB).

II. MATERIALS AND METHODS

A. Collection of plant samples

Young Leaf samples showing yellow vein mosaic symptoms were collected from three months old okra plants (Variety TV-8) growing in research field, Agriculture research station, Thirunelveli, Jaffna, Sri Lanka in May, 2015. Symptomless leaf samples were collected from plants which were grown in insect-proof cages. The leaf samples were dried by keeping inside paper towels.

B. DNA extraction

DNA extraction was done using above leaf samples. Fine powders of the leaf samples were obtained separately by grinding a whole leaf in liquid nitrogen using pre-chilled sterilized mortar and pestle. The aliquots (20 mg) of the fine powder were taken in 1.5 ml Eppendorf tubes separately. Total DNA was extracted using five different protocols including two modified methods which were introduced in this study (Table 1).

Table 1: DNA extraction protocols used in the study

No.	Protocol	Source
1	Cetyl trimethylammonium bromide (CTAB) method	Doyle and Doyle (1987)
2	An improved method of DNA isolation for mucilaginous plant	Ghosh *et al.* (2009)
3	DNeasy plant mini kit	QIAGEN, Germany
4	Modified method I	Proposed in the current study
5	Modified method II	Proposed in the current study

The protocols 1-3 were carried out as described by the authors or as in manufacturer's guide line and the protocol 4 and 5 are given below. All the chemicals used in the extraction procedures were purchased from Sigma-Aldrich, USA. All the extraction protocols were tested twice.

Protocol 4: Modified method I

This method was developed by modifying the method described by Ghosh *et al*. (2009). An aliquot of 5 ml of extraction buffer (100mM Tris–HCl, 10mM EDTA, 1.4M NaCl, 2% CTAB, 0.2% β-mercaptoethanol and 1% Polyvinylpyrrolidone (PVP), pH 8) was taken in 15 ml falcon tube and pre-warmed at 65 °C. 20 mg of leaf powder was added into the pre-warmed buffer and incubated at 65 °C for 30 min. During incubation period, the suspension was mixed 4 to 6 times by inverting the tubes gently. For removing the organic contaminants, 0.6 volume of chloroform:iso-amylalcohol (24:1) was added into the tube and mixed thoroughly for 1-2 min, to form an emulsion. The samples were centrifuged at 4000 × *g* for 10 min at room temperature and the upper aqueous phase was transferred to a fresh tube. Two-thirds of volume of cold iso-propanol was then added and mixed well by inverting the tube gently. The nucleic acid was collected as pellet by centrifuging at 4000 × *g* for 10min. The pellet was air-dried and re-suspended in 1 ml of TE buffer. The solution was transferred to a fresh 2 ml micro-centrifuge tube and treated with RNase at 37°C for 10 min in heating block. Then equal volume of chloroform: iso-amylalcohol (24:1) was added and mixed thoroughly to form an emulsion. The samples were centrifuged at 16,500 × *g* for 5 min at room temperature and the upper aqueous phase was transferred to a fresh tube. A double volume of absolute ethanol was added into the above aqueous phase and mixed gently by inverting the tube to precipitate the DNA. After centrifugation at 4000 × *g* for 10 min at 4 °C, the DNA pellet was washed with washing buffer, composed of ethanol (76%) and ammonium acetate (10 mM). Finally the air-dried DNA pellet was dissolved in 75 µl of TE buffer.

Protocol 5: Modified method II

The second modified method was developed by combining CTAB method and commercial DNA extraction kits. The procedure was similar to protocol 4 up to precipitation of nucleic acid by adding chilled iso-propanol. The resulting pellet was used for further extraction using DNeasy plant mini-kit using manufacturer's instruction.

C. Measuring the quality and quantity of extracted total DNA

To measure the DNA concentration, 1.5 µl of extracted DNA of each sample was loaded in Nanodrop spectrophotometer (Nanodrop 2000, Thermo scientific) and the concentration was measured at 260 nm wave length. The quality of the extracted DNA was measured based on the reading that obtained from A260/A280 nm and A260/A230 nm.

D. Quantification of plant DNA in the extracted total DNA using qPCR

The plant DNA in each extracted DNA sample was quantified and compared in relation to amount of plant actin gene. Pair of primers ACT2 F and ACT2 R (Table 2) amplifying a 68-bp genomic fragment of the *ACT2* gene (Che *et al*., 2010) was used in this experiment.

Quantification was done by absolute quantification method. The standard curve was prepared with known concentration of plant DNA which extracted from symptomless leaf samples. 50 ng/µl DNA extracted from leaf of virus free okra were tenfold serially diluted up to the final concentration 0.005 ng/µl. For each reaction 20 µl reaction mix was prepared by adding 10 µL SYBR Green mix (PCR BIOSYSTEM, UK), 1.5 µl of plant primer (5 µM) ACT2, 1 µl template DNA and rest of the volume was adjusted with nuclease free water. Quantity of *ACT2* gene in each DNA test sample (extracted using different protocols) was measured in the same reaction plate (96 well PCR plate) by adding 1µl of each DNA samples. No template control (NTC) reactions were carried out with nuclease-free water instead of DNA samples. The qPCR reaction was carried out in duplicates under conditions optimized for the primer (95 °C for 10 min and 40 cycles of 60 °C for 1 min, 95 °C for 15s) using a Step One Plus Real Time PCR system (Applied Biosystems, USA). At the end of the reaction a melting curve analysis was performed from 60 to 95 °C, with an increment of 0.5 °C at 10 s intervals.

E. Detection of virus DNA in extracted total DNA using end point PCR

The extracted total DNA was used as template in PCR for amplification of a segment of begomovirus DNA-A and whole genome of betasatellite associated with OYVMD. Primer pair MKBEGF4 and MKBEGR5 (Venkataravanappa *et al.*, 2012) was used to amplify 1.3 kilobase partial DNA-A and the universal primer pair β01 and β02 (Briddon *et al.*, 2002) were used to amplify the betasatellite DNA (Table 2).

Table 2: List of primers used in end point PCR and qPCR reactions

Primer	Target DNA	Primer sequence (5' to 3')
β01	Beta satellite DNA	GGTACCACTACGCTACGCAG CAGCC
β02		GGTACCTACCCTCCCAGGGG TACAC
MKBEGF4	Segment of DNA-A of Begomovirus	ATATCTGCAGGGNAARATHT GGATGGA
MKBEGR5		TGGACTGCAGACNGGNAAR ACNATGTGGGC
ACT2 F	Segment of actin gene of plant	CTTGCACCAAGCAGCATGAA
ACT2 R		CCGATCCAGACACTGTACTT CCTT

A 20 µl of PCR reaction mix was prepared by adding, 10 µl of PCR readymade mix (PCR BIOSYSTEM, UK), 1 µl of each primer (10 µM), 3 µl of DNA template (10 ng/µl) and 5 µl of nuclease free water. The reactions were carried out in a thermal cycler (Veriti®, Applied Biosystem, USA). For the partial amplification of DNA-A, the cycling conditions were maintained as follows; an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing temperature at 60 °C for 45 s and extension at 72 °C for 1 min with a final extension for 5 min at 72 °C. Similarly, the full length of betasatellite DNA was amplified with following PCR conditions; with initial denaturation at 94 °C for 5 min followed by 35 amplification cycles with melting at 94 °C for 1 min, annealing temperature at 58 °C for 1 min, extension for 1.5 min at 72 °C and final extension at 72 °C for 5 min. For both reactions, the optimum annealing temperature was determined by carrying out the temperature gradient PCR reaction with temperature ranging from 52 °C to 62 °C.

The PCR products were mixed with loading dye and run on 1.5% agarose gel at 90 volts for 30 min. Hyper-ladder I (Bioline, United Kingdom) was used as standard. The gel was stained with ethidium bromide and viewed on a Gel documentation system (UVP ChemiDoc-It TS2 Imager, United Kingdom).

F. Data analysis

Absolute quantification of plant DNA was determined by running the default settings of the StepOne™ Real-Time PCR Software Version 2.3 (Life technologies, USA) on the StepOne Plus™ Real-Time PCR systems (Applied Biosystems USA). The data were subjected to ANOVA in IBM SPSS Statistics for Windows, Version 21.0.

III. RESULTS

Five different DNA extraction protocols were tested to find out the protocol which can yield good quality and quantities of DNA extracted from okra leaves affected by OYVMD. The protocol 4 (modified method I), one of the methods that we introduced in this study, yielded higher concentration of DNA with good quality (Table 3); the average concentration of extracted total DNA was 85.8 ng/µl and the ratios A260/A280 and A260/A230 were 1.8 and 2.1 respectively. The total DNA extracted from 100 mg of the sample was 32.1 µg.

All other extraction methods, except protocol 5 (modified method II), produced a moderate yield; the concentration of the extracted total DNA ranged from 31 ng/µl to 50 ng/µl. However, the nano-drop reading for the quality measure of those DNA samples was not in the acceptable range in most of the samples. The protocol 5 (modified method II) yielded comparatively lowest quantity and quality DNA than other methods tested in this study (Table 3).

Table 3: Nano-drop spectrophotometer reading for the quantity and quality of total DNA extracted from leaf samples of okra

Methods	Concentration (ng/µl)	Total yield (µg/100 mg)[b]	Ratio (A260/A280)[c]	Ratio (A260/A230)[d]
Protocol 1: CTAB method	39.2 ± 2.4a	14.7 ± 1.3	1.6 ± 0.09	1.6 ± 0.02
Protocol 2: An improved method for mucilaginous plant	49.1 ± 2.9	18.4 ± 1.5	1.8 ± 0.07	1.5 ± 0.07
Protocol 3: DNeasy plant mini kit	31.3 ± 1.5	11.7 ± 0.8	1.6 ± 0.07	2.1± 0.12
Protocol 4: Modified method I	85.8 ± 2.1	32.1 ± 1.1	1.8 ± 0.05	2.1± 0.17
Protocol 5: Modified method II	16.3 ± 2.0	6.1 ± 1.0	1.7 ± 0.08	2.4 ± 0.10

[a] Mean value of three biological replicates with standard deviation from mean
[b] Amount of DNA yield in 100 mg leaf powder
[c] Acceptable reading is 1.8
[d] Acceptable range is 2-2.2.

qPCR assay for the quantification of plant DNA in relation to the quantity of actin gene showed results similar to nano-drop quantification in most of the samples (Figure 1). The quantity of the plant DNA in 1 µl of total DNA extracted according to the protocol 4 was 15.45 ng. It is significantly greater than the quantity of plant DNA in the DNA extracts of rest of the protocols. In protocol 2 and protocol 1 the amount of plant DNA was 8.30 ng and 5.81 ng respectively in 1 µl of total DNA samples. The nano-drop reading of DNA quantity for protocol 3 and protocol 5 contradict from qPCR quantification. The quantity of DNA obtained in protocol 3 was twofold higher than obtained in protocol 5. But in qPCR no significant variation was observed. However, in both type of quantifications the amount of DNA in protocol 3 and protocol 5 was comparatively lower than the rest of the protocols.

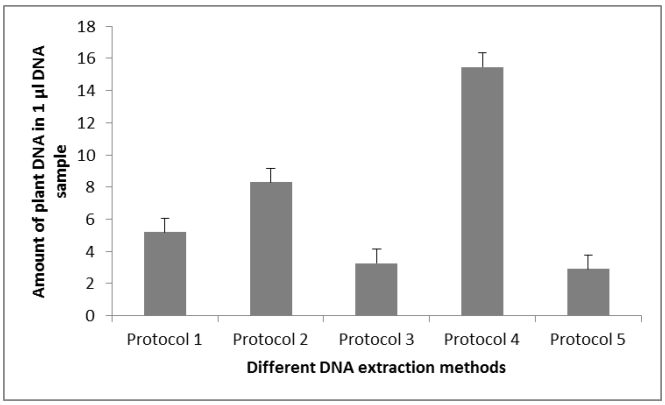

Figure 1. Amount of plant DNA in relation to the amount of actin gene copies in 1 µl total DNA extracted by five different methods. Protocol 1- CTAB method, Protocol 2- An improved method of DNA isolation for mucilaginous plant, Protocol 3- DNeasy plant mini kit, Protocol 4- Modified method I, Protocol 5- Modified method II. S. E. = 0.87.

End-point PCR was carried out to detect the beta-satellite DNA and DNA-A of OYVMV in the extracted total DNA samples. Presence of DNA bands with expected size (about 1.3 kilobase pair) in gel image indicates that all the tested extracts contain virus DNA (Figure 2). However, the intensity of the bands was not similar in all tested samples, which show variation in quantity of DNA in the tested samples. The bright band indicates higher copy number of the virus DNA and faint band indicates lower copy number of the virus DNA. Bright bands were observed in DNA samples extracted using protocol 1, protocol 2 and protocol 4 for both beta-satellite and DNA-A of OYVMV and less faint bands in DNA samples extracted using protocol 5. However, the results obtained for the DNA extracted in protocol 3 was not consistent; a brighter band was noticed for the amplification of DNA-A, but less bright band was for the beta-satellite DNA.

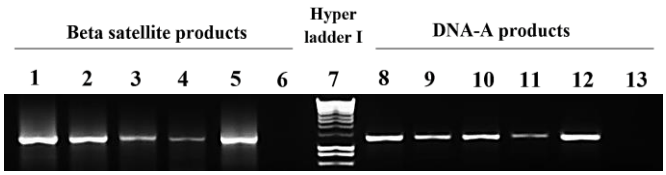

Figure 2. Comparison of different DNA isolation methods based on PCR amplified products of beta-satellite (1-6) and partial DNA-A (8-13). Amplification was detected in all samples with beta-satellite specific primers: (1) modified method I, (2) CTAB method. (3) DNeasy plant mini kit, (4) modified method II, (5) an improved method of DNA isolation for mucilaginous plant. Amplification was detected in all samples with DNA-A specific primers: (8) modified method I, (9) CTAB method. (10) DNeasy plant mini kit, (11) modified method II, (12) an improved method of DNA isolation for mucilaginous plant. No amplification was noticed in non-target control (6, 13).

IV. DISCUSSION

Most of the Malvaceae family members, including okra, have mucilaginous substances in their leaves and other plant parts. The mucilaginous substances greatly interfere in DNA extraction from these plant parts. In the present study, five different DNA extraction methods were tested and analysed to find out a best DNA extraction method that can yield higher quality and large quantity of DNA from okra leaves with the major aim to detect virus DNA. A modified DNA extraction method which was developed in this study (Protocol 4 - Modified method I) produced better quality and quantity of DNA, in addition, the extracted total DNA had large quantities of virus DNA.

Earlier, Ghosh *et al.* (2009) developed a modified CTAB method and recommended for the DNA extraction from highly

mucilaginous plants including okra. However, the protocol has some negative aspects such as using a highly toxic phenol in the extraction, the volume of reagents or buffers are relatively high and it takes too long and hard to re-suspend the DNA pallet in NaCl. In the present study, the modified method 1 is a modified version of the method described by Ghosh *et al.* (2009). However, the quantity of the DNA extracted in modified method 1 is twofold higher than the quantity of DNA extracted in the method described by Ghosh *et al.* (2009) (Table 3). The quality of the DNA extracted using the method described by Ghosh *et al.* (2009) was comparatively lower than the quality of DNA extracted in modified method 1; this might be due to the contamination of some amount of proteins and great amount of carbohydrates and phenolic compounds (Abdel-Latif and Osman., 2017).

Addition of PVP enhances the removal of secondary plant metabolites, especially polyphenolic compounds. The PVP binds with phenolic compounds and helps in their removal (Rezadoost *et al*., 2016). The superfluous quantities of cellular proteins were managed by twice extended treatment with chloroform-isoamyl alcohol. In addition to the removal of proteins, this treatment also helps to remove different coloring substances such as chlorophyll, pigments, and dyes (Azmat *et al*., 2012).

Phenol: chloroform extraction was not included in modified method 1 to avoid potential health hazards. Phenol is irritating to the skin, rapidly absorbed through the skin. Special protections are needed to handle this chemical. Therefore, nowadays researchers try to avoid using phenol in DNA extraction protocols.

In the method proposed by Ghosh *et al*. (2009), the crude nucleic acid pellet was dissolved in 1M NaCl. It took more than 30 min to partially re-suspend the pellet, therefore, it is time consuming and tedious when handling several samples at a time. In modified method 1, TE buffer was used to dissolve crude nucleic acid pellet; the pellet dissolved quickly, within 3 to 5 min. This modification did not affect the quantity and quality of the extracted DNA.

Mostly researchers use RNase to remove RNA that co-extracted with DNA. Studies have proved that RNase removing substantial quantities of DNA under common experimental conditions (Dona and Houseley, 2014). Therefore, prolonged treatment with RNase may also affect the quality and quantity of the DNA. Therefore, the RNase incubation time was reduced to 10 minutes. Dona and Houseley (2014) has proved that RNase treatment followed by phenol:chloroform purification step, as described in Ghosh *et al*. (2009), significantly reduced the yield of the DNA extraction.

Even though both CTAB method (Doyle and Doyle, 1987) and DNeasy plant mini kit method produced a moderated quantity of total DNA, the ratio of A260/A230 or A260/280 was greatly low. This could be due to the contamination of carbohydrates and phenolic compounds. In order to reduce these contaminants, in modified method 1 chloroform-isoamyl alcohol extraction was carried out twice.

The detergent CTAB can be used to isolate highly polymerized DNA from plant. It produces the conformational change in the DNA from random coil to compact globule, making DNA precipitation more effective. CTAB and chloroform:isoamyl alcohol mixture separates contaminants into the organic phase and nucleic acid into the aqueous phase (Doyle and Doyle, 1987). But, many plants have very high levels of secondary metabolites, including lipids, phenolic compounds and viscous polysaccharides. These can be removed by doing further processing, commonly with organic solvents, such as phenol or other toxic compounds (Pedersen *et al.*, 2006; Bashalkhanov and Rajora, 2008; Bellstedt *et al.*, 2010). The phenolic compounds are powerful oxidizing agents and bind covalently to extracted DNA making it useless for most of the molecular manipulation. Thus, if they are not removed properly, then they hinder subsequent downstream assays including PCR (Porebski *et al*., 1997; Angeles *et al.*, 2005).

Using commercial DNA extraction kits is easier, faster and relatively less hazardous than the conventional extraction methods. The extraction using commercially available kits consist of comparatively few steps for the completion of the entire extraction process, but it is expensive. For experiments with large scale DNA extraction and screening, the cost of the kits gives extra burden, especially to the researchers in developing countries. Conversely, conventional methods are cheaper even though they consume too long for extraction steps. The average completing time for the modified method1 was approximately 2 ½ hours, handling 10 samples at the same time. In overall, the results indicated that the DNA extraction using modified method 1 is advantageous over the other conventional methods with respect to the time, yield of the DNA and reproducibility by PCR.

There have been numerous reports of extraction of begomoviral DNA from wide range of plant species rich in mucilage and polysaccharides including okra (Jose and Usha, 2000), Bixa (Echevarria-Machado *et al.*, 2005), Sedum (Barnwell *et al.*, 1998), cotton (Mansoor *et al.*, 1999), forest plants (Scott and Playford, 1996) and legumes (Rouhibakhsh *et al*., 2008). Besides their specificity towards a particular plant species, most of these protocols are either time consuming or involve some additional reagents such as polyethylene glycol and silica which make these methods expensive and cumbersome.

Based on the findings of this study, the modified method 1 is a highly suitable method for the extraction of DNA from okra, because of higher yield and good quality of the extracted DNA. The DNA extracted in this method is highly useful in

downstream applications such as PCR based detection, identification and quantification of virus DNA.

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