International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2015): 78.96 | Impact Factor (2015): 6.391

Extraction of High Quality DNA from Mucilaginous Plants with a New Improved Method, Suitable for Detection of Geminiviruses and Downstream Applications

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Abstract: ***High quality genomic DNA extraction from higher plants is interfered by the presence of secondary metabolites, which reduces the yield and quality of the DNA and the downstream processes such as DNA amplification, restriction digestion and cloning. We describe an alternative protocol for genomic DNA extraction from fresh and dry mucilaginous plant leaves that is amenable to PCR-based genetic analysis. It is relatively simple quick, rapid, less time consuming and cost effective method for DNA isolation from leaves of mucilaginous plant using modified cetyl trimethylammonium bromide (CTAB) method for extraction of DNA from leaf materials. DNA isolated using this method showed consistency in yield and compatibility with PCR for detection of geminiviruses from different mucilaginous plant species. The method was compared for efficacy with other reported methods and it was found to be superior over the existing methods described for isolation of DNA from mucilaginous hosts. Thus the method described could be used on a wider scale for reliable and consistent detection of geminiviruses from mucilaginous hosts for characterization and variability study.***

Keywords: Geminivirus, Malachra sp., DNA extraction, mucilaginous plants

1. Introduction

A large number of not only crop plant but also much wild plants are susceptible to infection by geminiviruses. The family *Geminiviridae* contains four genera *viz* Mastrevirus, Topocuvirus, Curtovirus and Begomovirus. Begomovirus is the largest genus of all viral taxonomy with respect to the number of species that it includes. In fact, currently 322 species have been recognized by the International Committee on Taxonomy of Viruses (ICTV) (http://www.ictvonline.org/virusTaxonomy.asp).

Geminiviruses contains one or two small circular single stranded DNA (approx.2.7-3.0 kbp). The worldwide expansion of agriculture has also resulted in the emergence and spread of numerous insect pests and diseases including many destructive viral diseases. Geminiviruses are insect-transmitted viruses that have emerged, over the past two decades and are one of the most economically important emerging and re-emerging viruses [1]. Isolation of good quality DNA is a prerequisite for the applications such as different molecular techniques when studying any organism or pathogen or diagnosis. Plants produce secondary metabolites that interfere not only with extraction of high quality genomic DNA but also with the subsequent reactions such as PCR and related genetic analyses [2][3]. Since the biochemical composition of plant tissues vary in different species, DNA isolation protocols need to be optimized for each species [4]. The family Malvaceae containing mucilaginous plant like *Malachra capitata*, *Hibiscus rosa-sinensis*, bhendi or okra (*Abelmoschus esculentus*) possess high amount of foliar mucilage. DNA isolation from mucilaginous plant is difficult due to the high content of polyphenols and polysaccharides which are found to be not only co-precipitate with the DNA but also involves in the degradation of DNA due to endonucleases that hampers restriction digestion of DNA, PCR amplification and cloning. There are many protocols for DNA preparations from various sources of tissue have been published over the last few decades. We have tried five different protocols for the isolation of DNA from mucilaginous plant *M. capitata* [5][6][7][8] but we found that most of the protocols are not very suitable for the isolation of DNA from *M. capitata*. With this background we have developed a simple, rapid, less time consuming and cost effective method for DNA isolation from leaves of mucilaginous plants such as Bhendi (*Abelmoschus esculentus*), *Jatropha gossipiifolia*, *Croton bonplandianus*, Jute (*Corchorus* sp.), *Sida* sp and *M. capitata*. The aim of our study was to develop a rapid and cost efficient method for extraction of genomic DNA from leaves of mucilaginous plant, gives pure viral DNA without ultra-purification.

2. Materials and Methods

Plant samples for DNA isolation: Initially, for standardization of the method, geminivirus infected *M. capitata* leaves with yellow mosaic typical of geminivirus symptoms were chosen as the model plant sample. After standardization, the method was validated with different mucilaginous plants samples with typical symptoms of geminivirus infection**.** All plant materials used in this study were collected from different parts of the North 24 Parganas, West Bengal, India. Plant materials used for this purpose were Bhendi (*Abelmoschus esculentus*), *Jatropha gossipiifolia*, *Croton bonplandianus*, Jute (*Corchorus* sp.), *Sida* sp and *M. capitata* infected with virus and showing typical geminivirus symptoms.

DNA extraction: To compare the efficiency of the current protocol, total DNA was isolated from geminivirus infected *M. capitata* samples obtained from North 24 Parganas, district of West Bengal state using following methods, *viz.*: total genomic DNA extraction done according to: **1)** cetyl trimethylammonium bromide (CTAB) method [8]; **2)** improved DNA extraction method [5]; **3)** Modified CTAB methods for extraction of DNA [6]; **4)** extraction with citrate buffer and alkali lysis, PEG precipitation [7]; **5)** new improved CTAB method in this study (procedure described as below) and **6)** extraction using Hipure™ plant genomic DNA miniprep purification kit (Himedia) (Fig.1).

(a) Reagent and chemicals required

•Trish-HCL pH 8.0 (1.0 M); EDTA pH8.0 (0.5 M); NaCl (5.0M); CTAB (20%); Chloroform: Isoamyl alcohol (24:1 v/v); Polyvinylpolypyrrolidone (PVP) and β-mercaptoethanol.

•**Extraction Buffer**: 2.5% (w/v) CTAB; 25 mM EDTA, pH 8.0; 100 mM Tris–HCl (pH8.0); 2 M NaCl, PVP 2% (w/v) (added immediately before use) and β-mercaptoethanol 0.4% (v/v) (added immediately before use).

• Chloroform: isoamyl alcohol (24:1).
• 70% Ethanol.
• Isopropanol.

(b) Protocol for Isolation of DNA

1. Ground the plant material in liquid nitrogen (100 mg fresh plant tissue).
2. Added 1 ml of freshly prepared extraction buffer.
3. Mixed and transferred the ground material to 2 ml polypropylene tube; incubated at 65°C for 30 minutes in a water bath with intermittent mixing by gentle swirling.
4. Added an equal volume of chloroform: isoamyl alcohol (24:1) and mixed by inverting the tubes several times.
5. Centrifuged at 12,000 g for 10 min. at 25°C.
6. Carefully transferred the aqueous phase into sterile polypropylene tube and 0.5 volume of ice cold isopropanol was added and mixed gently.
7. Incubated the tubes at -20°C for 1 hour.
8. Centrifuged the tubes at 12,000 g for 10 min at 4°C.
9. Discarded the supernatant and washed the pellet with 70% ethanol, air dry and resuspended in 30 µl deionized distilled water and used for downstream application.

Primer for PCR amplification:

For the detection and amplification of geminivirus, DNA was isolated from different mucilaginous plants, which showed specific geminiviral symptoms. From extracted DNA, PCR reaction were carried out in 0.2 ml polypropylene PCR tubes, by using geminivirus DNA-A specific degenerate primer pair [9] amplifying approximately 760 bp of sequence representing part of AV1, AC3 and AC2 genes (Fig. 2b) which are partially overlapped to each other. Each 25µl reaction mixture contained 12.5 µl master mix (BlackBio), 20 pmol oligonucleotide primer and 30 ng of template DNA. The reaction were subjected to initial denaturation at 94ºC for 5 min, followed by 30 amplification cycles, each consisting of 30 sec at 94ºC (denaturation step), 45 sec at 48ºC (annealing step) and 1 min at 72ºC (extension step) with a final extension of 7 min at 72ºC. The PCR amplified products were separated on 1% (w/v) agarose gel.

3. Result and Discussion

For several molecular biology applications, isolation of high quality genomic DNA is pre-requisite but due to presence of secondary metabolites in mucilaginous plants, it creates hindrance in various downstream applications. The secondary compounds may hamper the DNA quality as well as many other downstream reactions to be carried such as restriction enzymes digestion, PCR amplification; ligation, cloning etc. may be inhibited. In our present study for the isolation of total DNA from geminivirus infected *M. capitata* plants, we encountered difficulties from the stage of cell lyses to DNA separation in the supernatant and subsequent reactions when following the procedures described by : 1) cetyl trimethylammonium bromide (CTAB) method [8]; 2) improved DNA extraction method [5]; 3) Modified CTAB methods for extraction of DNA [6]; 4) extraction with citrate buffer and alkali lysis, PEG precipitation [7]; and 5) extraction using Hipure ™ plant genomic DNA miniprep purification kit (Himedia) (Fig.1). Major problems encountered were low of yield DNA and presence of the impurities. The amount of DNA obtained from 100 mg of geminivirus infected *M. capitata* leaves by various extraction methods was determined spectrophotometrically and checked by gel electrophoresis. We have developed a new modified and improved CTAB method as described above. In this study, we have found that the DNA yield was comparably more than the other methods using the same amount of leaf tissue, purity of the DNA was better than other and the method is less time consuming and cost effective than other methods (Table 1). Among the other methods, DNA obtained by a commercial kit yielded the least amount of DNA due to clogging of the column with mucilaginous substances present in the plant samples. Our protocol involves isopropanol precipitation of DNA initially at room temperature. Moreover, the procedure also eliminates the necessity of use of phenol, which makes the method less hazardous. Further, the addition of high concentration of PVP and β-mercaptoethanol were helpful in removing the polyphenols from mucilaginous plant samples. The problem arising from the presence of high levels of polysaccharides was overcome by using NaCl at a higher concentration. By using the above protocol a fairly high yield of high quality DNA was obtained for each plant (Fig.1a). The amount of DNA recovered per gram of plant material was sufficiently high and this recovery was possible from various plant parts as well (Table 2). The current method proves well for the isolation of the DNA from the plants we tested and may be used for the isolation of DNA from any other mucilaginous plants. The expected size of ~760 bp PCR amplification from DNA-A, with pair of primers used by Roy et al., 2015, also indicated the presence of DNA-A of geminivirus into the test plants (Fig.2b). The purity and clean nature of

DNA samples was confirmed through complete digestion by the restriction enzyme *Eco*RI (4 units/µg DNA) by incubating the reaction tubes at 37ºC for 30 min and also by A260/A280. This indicated that isolated DNA can be used in downstream applications such as cloning experiments as well as DNA fingerprinting.

Thus, the new methods could be used on a wider scale as a simple and cheaper method of DNA isolation suitable for characterization of geminiviruses infecting many other mucilaginous hosts and other downstream applications.

4. Conclusion

High quality DNA is pre-requisite for the molecular study of the plant viruses. The presence of secondary metabolites or polysaccharide derivatives has been found to create hindrance for the isolation of the high quality DNA and different downstream processes. Moreover, the chemotypic heterogeneity among species may cause interference in optimal DNA yields with a single DNA isolation protocol. Most of the DNA extraction protocols need large amount of starting material and extraction are done in large volume of the buffer. The present method require small amount of starting tissues for successful extraction and large numbers of samples could be processed at a time in parallel. The present protocol could be used for miniprep as well as maxiprep DNA isolations from different mucilaginous plants. In this method also do not need RNase treatment for DNA isolation making it more cost effective and less time consuming without compromising the quality, quantity and purity of the DNA. The aim of this study is to develop simple, fast and cost effective DNA isolation protocol from the mucilaginous containing plants such as *M. capitata*. Usually the cumbersome procedures makes the DNA prone to degradation and most of the protocols are not suitable for all the plants. The present protocol is simple, fast and cost effective and can be done is laboratories with minimum facilities. The protocol described here is thus useful for obtaining good quality viral DNA, from highly mucilaginous plants such as *Malachra capitata* and related genera and DNA isolation can be performed in small volume of centrifuge tube.

Acknowledgement

Authors would like to thank Department of Science and Technology, New Delhi for SERB Project Grant No. SR/FT/LS-165/2012 and financial assistant from University Grant Commission (UGC), New Delhi to Mr. Buddhadeb Roy, Senior Research Fellow (Rajiv Gandhi National Fellowship) in the form of fellowship is greatly acknowledged.

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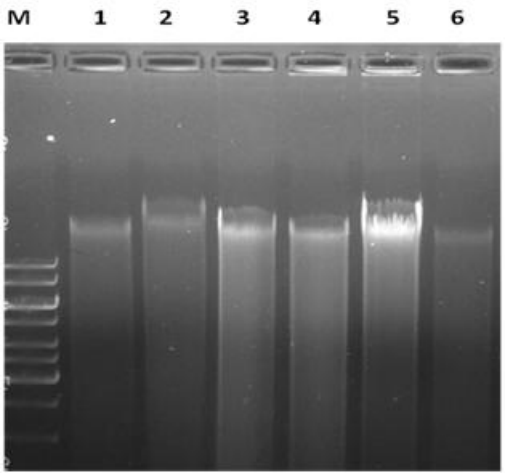

Figure 1: Gel images of total DNA isolated through different protocol from leaves of *Malachra capitata*. M: 1kb DNA marker; lane 1: Doyle and Doyle, 1987; lane 2: Sharma and Purohit, 2012; lane 3: Ghosh et al., 2009; lane 4: Jose and Usha 2000; lane 5: Improved CTAB method in this study; lane 6: Hipure ™ plant genomic DNA miniprep purification kit (Himedia).

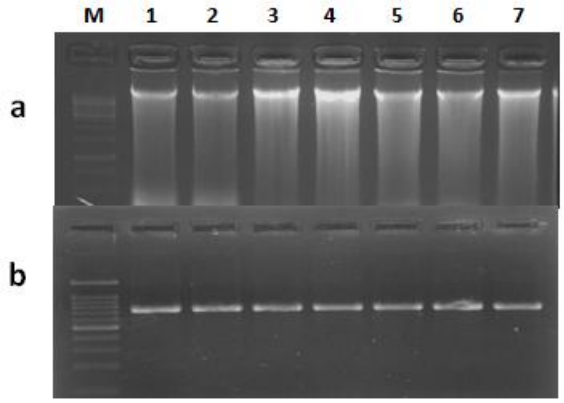

Figure 2: Isolated DNA from seven plant species resolved on 1% agarose gel. **(a)** Purified DNA; **(b)** PCR of the DNA samples amplified with geminivirus specific primer pair; **M**:marker; **lane 1**, Bhendi (*Abelmoschus esculentus*); **lane 2**, *Jatropha gossipiifolia* ; **lane 3**, *Croton bonplandianus* ; **lane 4**, *Malachra capitata* ; **lane 5**, Jute (*Corchorus sp*.); **lane 6**, *Sida sp.;* **lane 7**, *Ludwigia sp.*

Table 1: DNA yield and purity obtained from *Malachra capitata* using various protocols:

No.	Protocol	Yield (µg/ml)	OD (260/280 ratio)	Reference
1.	Protocol 1	120-150	1.0-1.2	Doyle and Doyle, 1987
2.	Protocol 2	225-287.5	1.1-1.3	Sharma and Purohit, 2012;
3.	Protocol 3	400-450	1.5-1.6	Ghosh et al., 2009;
4.	Protocol 4	150-312	1.3-1.4	Jose and Usha 2000
5.	Protocol 5	500-687.5	1.7-1.85	Improved CTAB method in this study
6.	Protocol 6	131-137.5	0.85-.0.9	Hipure ^{TM} plant genomic DNA miniprep purification kit (Himedia)

Table 2: DNA yield and purity obtained from various mucilaginous leaves using new improve protocols:

No.	Plant (Family)	Yield (µg/ml)**	OD (260/280 ratio)**
1.	Bhendi (*Abelmoschus esculentus*) (Malvaceae)	662.5	1.65
2.	*Jatropha gossipiifolia* (Euphorbiacea)	612.5	1.75
3.	*Croton bonplandianus* (Euphorbiacea)	537.5	1.72
4.	*Malachra capitata* (Malvaceae)	600	1.8
5.	Jute (*Corchorus sp.*) (Malvaceae)	562.5	1.8
6.	*Sida sp.* (Malvaceae)	550	1.76
7.	*Ludwigia sp.* (Onagraceae)	575	1.76

******Results expressed is mean of three readings