ISSN 1996-0700

Asian Journal of Biotechnology

http://knowledgiascientific.com

Knowledgia SCIENTIFIC
A Place to Publish Outstanding Research

Asian Journal of Biotechnology 7 (2): 80-87, 2015
ISSN 1996-0700 / DOI: 10.3923/ajbkr.2015.80.87

An Efficient DNA Extraction Protocol for Successful PCR Detection of *Banana bunchy top virus* from Banana Leaves

Shivangi Johari and S. Majumder
Department of Biotechnology, SET, Sharda University, Greater Noida, India

Corresponding Author: Shivangi Johari, Department of Biotechnology, SET, Sharda University, Greater Noida, India

ABSTRACT

Banana bunchy top virus (BBVT) is the most threatening disease of banana all over the world. Control of BBTV involves the use of disease free planting material and prevention of infection. Its accurate detection is one of the functional approach to control it by eradication and exclusion. PCR is the most efficient mode of indexing but high-quality of DNA is one of the most essential factor for a successful amplification by PCR. In this study, we have developed a highly efficient low cost and rapid DNA extraction method for banana tissue which could yield good quantity of DNA free from protein and polysaccharide contamination. The DNA extracted by this method could be successfully amplified by PCR. In addition, the protocol could be completed within ~2 h.

Key words: Non phenol-chloroform extraction, polyphenols, viral diagnosis

INTRODUCTION

Banana and plantains are grown in about 120 countries in mixed cropping systems by small holders and occasionally in monoculture (INIBAP., 1992). It is known that banana is the major staple food crop for approximately 400 million people (INIBAP., 2000). Banana is a major export crop in many of the tropical and subtropical areas of the world (Anonymous, 1992). Banana plants are grown in 120 countries worldwide - more than any other fruit crop (Arias *et al*., 2003). According to Food and Agriculture Organization (FAO), about 102 million t of bananas were produced worldwide in 2012. India leads the world in banana production with an annual output of about 2.75 million t (FAO., 2013). Loss of banana production by pests and diseases is one of the major problems worldwide. There are many economically important diseases of banana caused by the pathogens that include bacteria, fungi, nematode and virus. The viruses, in comparison to other pathogens, are more devastating for banana plantation. And among viruses, *Banana bunchy top virus* (BBTV) is the most devastating and therefore the most economically important virus. Banana bunchy top disease (BBTD) is considered most destructive of all the viral diseases affecting banana worldwide, which can cause yield loss even up to 100% (Dale, 1987). The BBTD is fast emerging as most destructive and widespread viral disease in banana plantations of the world (Furuya *et al*., 2005). As, there is no report of natural resistance to this virus, the only way to effectively control or escape this virus is through production of virus free planting material. The production of virus free planting material, primarily and essentially requires a sensitive virus detection technique.

Detection of BBTV in banana plant has been attempted using several methods such as Enzyme Linked Immunosorbent Assay (ELISA), Immunosorbent Electron Microscopy (I-SEM) and

Polymerase Chain Reaction (PCR) (Su *et al*., 2003; Furuya *et al*., 2005). Currently, PCR is a common technique routinely used in diagnostic test. It is preferred over other methods for its accuracy and rapidity (Elnifro *et al*., 2000).

The PCR based diagnostic test usually require preparation of high quality DNA (Souza *et al*., 2012), which is done either by multistep extraction, phenol chloroform precipitation or commercial kits. Banana plant is known to have high amount of polyphenols and polysaccharides, which interfere during usual DNA extraction process and usually remain as impurities in the DNA template preparations (Bryant, 1997). The phenolic compounds get oxidized and irreversibly bind to nucleic acids and various proteins at the time of tissue homogenization (Loomis, 1974; Aljanabi *et al*., 1999). High content of polysaccharides make DNA difficult to amplify in the PCR due to its viscous nature, inhibit activity of *Taq* polymerase and affect activity of restriction enzymes (Porebski *et al*., 1997). Further, usual DNA extraction requires organic solvent such as phenol and chloroform which are hazardous (Sambrook and Russell, 2001). Therefore, there is a need to standardise an efficient and safe nucleic acid extraction method specifically adapted to banana plants. The method need to be evaluated for its efficiency and cost effectiveness.

In this study we have developed two simplified and economical DNA extraction method and compared them with two other DNA extraction protocol. The most efficient method was then chosen as the method of choice for extracting DNA intended for PCR based diagnosis of BBTV.

MATERIALS AND METHODS

Source of sample: The leaf sample from naturally infected banana plants showing typical BBTD symptoms were collected from Raipur, India and maintained in experimental net house.

DNA extraction protocols

Protocol A: DNA extraction using SDS and LiCl: Hundred milligram of infected leaf were homogenized in liquid nitrogen and mixed with 500 µL of extraction buffer containing 100 mM Tris pH 8.5, 10 mM EDTA, 300 mM lithium chloride, 1.5% SDS and transferred into a microcentrifuge tube. The tube was incubated at 65°C for 10 min, 500 µL of 6 M potassium acetate was added to it. The tube was kept in ice for 10 min and then centrifuged at 10,000 rpm for 10 min. The supernatant was collected in a fresh tube and mixed with one volume of chilled isopropanol. The tube was further incubated at -20°C for 1 h. After centrifugation at 10,000 rpm for 10 min, the supernatant was discarded and the pellet was washed with alcohol. The pellet was air-dried and resuspended in 300 µL of sterile water. The solution was then mixed with 30 µL of 3 M sodium acetate and 600 µL of ethanol. The tube was again incubated at -20°C for 1 h. After centrifugation at 10,000 rpm for 10 min, the supernatant was discarded and the pellet was washed with alcohol. The washed pellet was air-dried and dissolved in 20 µL of sterile water.

Protocol B: DNA extraction using TritonX-100 and LiCl: Hundred milligram of infected leaf were homogenized in 500 µL of extraction buffer containing 100 mM Tris pH 8.5, 10 mM EDTA, 300 mM lithium chloride, 1% TritonX-100 and transferred into a microcentrifuge tube. The tube was incubated at 65°C for 10 min, 500 µL of 6 M potassium acetate was added to it. Next, the tube was kept in ice for 10 min and then centrifuged at 10,000 rpm for 10 min. The supernatant was collected in a fresh tube and mixed with one volume of chilled isopropanol. The tube was further incubated at -20°C for 1 h. After centrifugation at 10,000 rpm for 10 min, the supernatant was discarded and the pellet was washed with alcohol. The pellet was air-dried and resuspended in

300 µL of sterile water. The solution was then mixed with 30 µL of 3 M sodium acetate and 600 µL of ethanol. The tube was again incubated at -20°C for 1 h. After centrifugation at 10,000 rpm for 10 min, the supernatant was discarded and the pellet was washed with alcohol. The washed pellet was air-dried and dissolved in 20 µL of sterile water.

Protocol C: DNA extraction using commercial kit: Total DNA was extracted from 50 mg tissue of leaves from infected and healthy plants, using DNeasy Plant Mini Kit (Qiagen Germany) as per manufacturer's protocol.

Gel electrophoresis: The quality of DNA was checked by gel electrophoresis. The sample were analyzed in 1% agarose gel prepared in 1X TAE buffer, with 2% ethidium bromide (0.5 µg mL^{-1}).

DNA quantification: DNA concentration (obtained by three different methods) was estimated by spectrophotometer (Shimadzu UV-240). The OD was taken at 260 nm for DNA and 280 nm for protein. A ratio of OD-260/280 nm was observed as an indicator of DNA purity. Absorbance was also taken at 260 and 230 nm, the ratio of A260/230 ensure that extracted DNA is free of polysaccharides.

Primer designing and synthesis: Specific primer to amplify replication initiation protein (Rep) of BBTV was designed using the replication initiation (Rep) gene sequences available at NCBI Genbank. Bioedit (7.0.7.1) (Hall, 1999) and CLUSTAL W (Thompson *et al*., 1994) was used to align the sequences and locate the conserved regions. The primers were synthesized by Sigma Aldrich (Bangalore).

PCR amplification: PCR was performed in a thermal cycler (MJ Mini, Biorad) using 25 µL reaction that contained 0.2 µmol each primer, 10 mM dNTPs (0.5 µL), 5U of Taq DNA polymerase (0.5 µL), 10 X reaction buffer (2.5 µL), 25 mM MgCl2 (1.5 µl), 5.0 µL total DNA and sterile water to make up the volume. The mixture was subjected to one cycle of denaturation at 94°C for 5 min, followed by 29 cycle of denaturation at 94°C for 45 s, annealing at 65°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 1 min. Amplicons were analyzed in 1% agarose gel prepared in Tris-acetate EDTA buffer containing 0.5 µg mL^{-1} ethidium bromide. The gel was visualized and photographed under UV illumination with an imaging system (Biorad XR documentation system).

Sequencing and sequence analysis: The amplified DNA product of BBTV was purified using PCR purification kit (Qiagen, Germany). Sequencing of PCR products were done at Chromous Biotech, Bangalore, India. Sequence obtained was subjected to BLAST analysis for deducing the sequence homology of nucleotides as well as amino acids. Bioedit (7.0.7.1) was used for converting the nucleotide sequence to amino acid sequence. The conserved domains in the predicted proteins were explored using conserved protein domain database (CDD).

Efficacy: To validate the protocols and test their efficiency, the PCR was performed with serially diluted DNA samples. PCR was performed at various dilutions to ascertain the maximum dilution at which the virus can be detected by these protocols. The dilution range was 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}.

Efficacy of the protocol was also examined by studying the shelf life of the extracted DNA over a period of four months. The best protocol was used for extracting DNA used for indexing symptomatic and asymptomatic field samples collected from three states viz., Assam, Chattisgarh and Kerala.

Further, total DNA isolated by protocol A and B was also used for amplification of full length BBTV components DNA-U3, DNA-S, DNA-C, DNA-M and DNA-N. An expected amplicon of ~1.1 kb was obtained for all five components. To confirm the identity of the amplified PCR product the amplicons were sequenced at Chromus Biotech, Bangalore, India.

RESULT

DNA purity: In this study, four different protocols followed for genomic DNA isolation was found to give satisfactory result in terms of DNA yield and purity (Table 1). The ratio of absorbance at 260 and 280 nm was found to vary for different protocols (Table 1). The ratio of absorbance at 260 and 280 nm was found to vary from 1.71 and 1.72, for protocol A and B, respectively, similar to the DNA extracted by commercial Kit which was 1.73.

PCR analysis: DNA extracted by all the three protocols produced an expected amplicon of 1.1 kb. DNA isolated by protocol A and B gave bands which were bright and reproducible (Fig. 1).

Analysis of sequence data: Sequencing of the PCR product resulted in ~1.1 kb long nucleotide sequence. The nucleotide sequence of this gene has a potential to encode a 206 amino acids residues. The amino acid sequence for the predicted protein shared identity of 99-100% with replicase protein of other BBTV isolates.

Efficacy of PCR: To test the efficacy of the protocols, PCR was performed with DNA serially diluted from 10^{-1} to 10^{-4} (Fig. 2). DNA obtained by protocol A and B were able to produce clearly

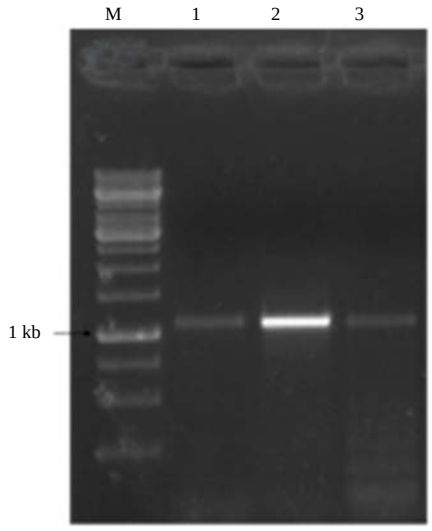

Fig. 1: PCR analysis of DNA isolated from three different protocols using specific Rep gene primer (1.1 kb). Lane M: Marker 1 kb, lane 1: PCR amplification of DNA isolated by protocol A, lane 2: PCR amplification of DNA isolate by protocol B, lane 3: PCR amplification of DNA isolated by protocol C

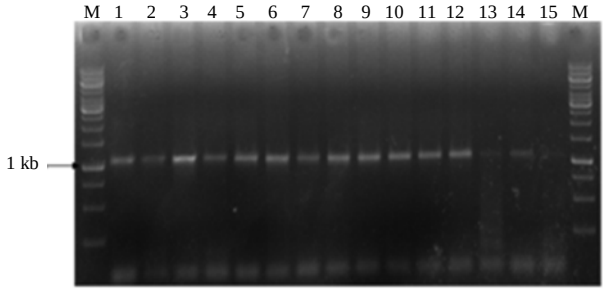

Fig. 2: PCR amplification of DNA Isolated by three different protocols at 10^{-1} to 10^{-4} dilution. Lane M: Marker 1 kb, Lane 1, 4, 7, 10, 13, DNA isolated by protocol A at 1, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilution, Lane 2, 5, 8, 11, 14, DNA isolated by protocol B at 1, 10^{-1}, 10^{-2},10^{-3} and 10^{-4} dilution, Lane 3, 6, 9, 12, 15, DNA isolated by protocol C at 1, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilution

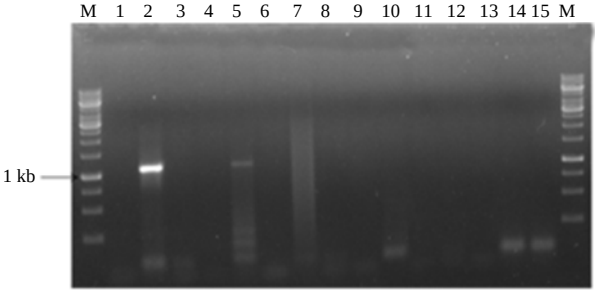

Fig. 3: PCR analysis of DNA isolated from protocol B for asymptomatic leaves. Lane M: Marker 1 kb, lanes 1, 3, 4, 6-15: Non amplification of DNA from asymptomatic leaves, Lanes 2 and 5: PCR amplification of DNA from asymptomatic leaves

Table 1: Absorbance at 260 and 280 nm for DNA obtained with three different extraction protocols

Methods	OD (260 nm)	OD (280 nm)	Ratio (260/280)
Protocol A	0.036	0.021	1.71
Protocol B	0.025	0.015	1.72
Protocol C	0.033	0.019	1.73

OD: Optical density

visible band at 10^{-2} and 10^{-3} dilution, in comparison to protocol C where visibility of band was low even at 10^{-2}. Sequence analysis of PCR product of other DNA components confirmed their identity.

The shelf life of extracted DNA by two protocols developed in this study, when examined over a period of four months showed that protocol B was still functional and better than protocol A. So, protocol B was used for extracting DNA used for indexing symptomatic and asymptomatic field samples collected from three states viz., Assam, Chattisgarh and Kerala. It was found that out of the 15 asymptomatic samples, 2 samples were tested positive (Fig. 3).

DISCUSSION

Banana is one of the most important crops for about 120 countries. In many of these countries viz., India, indexing of banana planting material is mandatory by law (MoA., 2003). PCR

is considered one of the most efficient modes of indexing (Mullis and Faloona, 1987) and high-quality of DNA is one of the most essential factors for a successful amplification by PCR (Ahmed *et al*., 2009). Isolation of highly purified plant DNA is difficult, particularly from plants like banana with high polyphenolic compounds (Couch and Fritz, 1990). Phenolic compounds are ubiquitous in vegetative tissue of banana which acts as an important part of the plant's defense system against pests and diseases, including root parasitic nematodes (Wuyts *et al*., 2006). But at the same time, the phenolic compounds hamper biochemical and molecular studies in banana, as isolation of pure DNA becomes difficult (Wuyts *et al*., 2006) because these polyphenols get oxidised resulting in quinonic compounds that cause browning of DNA preparations, often damage the DNA and also render the DNA inaccessible for some enzymes (Weising *et al*., 2005). Protocol for extracting PCR appropriate DNA, may include different filtration process and treatment with various specific reagents that renders the whole process particularly expensive to be used for routine DNA extraction in indexing labs. Therefore, it was imperative to establish an inexpensive and less time-consuming protocol that could be used by diagnostic labs.

In protocol A and B, we have tried to remove the cellular membranes with SDS and TritonX-100 respectively. Both these reagents are mild detergents and hence surfactant. The SDS is also chaotropic in nature which aids in disrupting proteins. In our protocol development, we have tried to exploit these properties of the reagents in removing the lipid and protein component of the cellular membranes. In both the protocols, EDTA is being used to sequester divalent cations such as Mg^{2+}. These ions act as cofactor for DNase nucleases and by chelating them we were able to prevent the action of nucleases. The high concentration of LiCl (0.3 M) used in both of our buffers helps in removing the remaining proteins and dissolve polysaccharides. The high salt concentration in presence of isopropanol also selectively precipitates the DNA. A second purification step is involved where we re-precipitate the DNA in presence of ethanol to remove any residual impurity. In this study three protocols (protocol A, B and C) to isolate DNA from banana leaf tissue has been used and their efficacy in molecular experiments has been compared. The two new protocols developed and optimized in this study yielded good quality DNA with a ratio of absorbance at A260/280 of ~1.7. This indicates that the DNA fraction was free of protein impurities and could be used for further applications. The presence of polysaccharide in the DNA sample inhibits enzyme activity (Fang *et al*., 1992) but DNA extracted by A and B showed a ratio of ~2.7 at A260/230 indicating the extracted DNA to be free of polysaccharides. This ratio is used as a secondary measure of nucleic acid purity. Successful amplification of BBTV Rep gene with DNA extracted by these methods further confirms our judgment. Both the protocols yielded good quantity of DNA ranging from 1.8-1.25 μg mL^{-1} for protocol A and B, respectively. In addition, both of these protocols could complete the extraction within ~2 h, proving their efficiency.

The commercial kit used for DNA extraction protocols though highly efficient are expensive and hence not practically viable to be used by indexing labs. In this study we have developed two economically viable, efficient and rapid DNA isolation method, which had comparable efficiency with commercial kits. Both protocol developed in this study were equally efficient but DNA from protocol B was able to amplify and detect BBTV in banana leaf even up to the dilution of 10^{-4} which was better than that achieved by the commercially available kit or protocol A (Fig. 2). Further, protocol B is cheaper than A and is at least 30 times cheaper than the kit. Thus, protocol B provides an efficient, rapid and inexpensive DNA extraction protocol for extracting DNA from banana tissue to be used for PCR.

ACKNOWLEDGMENTS

Authors would like to thank Department of Biotechnology for providing financial support to carry out the work. They are also thankful to Sharda University, Greater Noida for providing infrastructure facility.

REFERENCES

Ahmed, I., M. Islam, W. Arshad, A. Mannan, W. Ahmad and B. Mirza, 2009. High-quality plant DNA extraction for PCR: An easy approach. J. Applied Genet., 50: 105-107.

Aljanabi, S.M., L. Forget and A. Dookun, 1999. An improved and rapid protocol for the isolation of polysaccharide- and polyphenol-free sugarcane DNA. Plant Mol. Biol. Rep., 17: 1-8.

Anonymous, 1992. Bananas, plantains and INIBAP. Annual Report, International Network for the Improvement of Banana and Plantain, Montpellier, France.

Arias, P., C. Dankers, P. Liu and P. Pilkauskas, 2003. The World Banana Economy 1985-2002. FAO, Rome, ISBN-13: 9789251050576, Pages: 86.

Bryant, J.A., 1997. DNA Extraction. In: Methods in Plant Biochemistry, Dey, P.M. and J.B. Harborne (Eds.). Vol. 10, Academic Press, San Diego, pp: 1-12.

Couch, J.A. and P.J. Fritz, 1990. Isolation of DNA from plants high in polyphenolics. Plant Mol. Biol. Rep., 8: 8-12.

Dale, J.L., 1987. Banana bunchy top: An economically important tropical plant virus disease. Adv. Virus Res., 33: 301-325.

Elnifro, E.M., R.J. Cooper, P.E. Klapper, A.C. Yeo and A.B. Tullo, 2000. Multiplex polymerase chain reaction for diagnosis of viral and chlamydial keratoconjunctivitis. Invest. Ophthalmol. Visual Sci., 41: 1818-1822.

FAO., 2013. FAOSTAT. Food and Agricultural Organization of the United Nations, Rome, Italy.

Fang, G., S. Hammar and R. Grumet, 1992. A quick and inexpensive method for removing polysaccharides from plant genomic DNA. BioTechniques, 13: 52-56.

Furuya, N., S. Kawano and K.T. Natsuaki, 2005. Characterization and genetic status of *Banana bunchy* top virus isolated from Okinawa, Japan. J. Gen. Plant Pathol., 71: 68-73.

Hall, T.A., 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acid Symp. Ser., 41: 95-98.

INIBAP., 1992. Annual report. International Network for the Improvement of Banana and Plantain (INIBAP), Montpellier, France.

INIBAP., 2000. Banana: Food for the poor. International Network for the Improvement of Banana and Plantain (INIBAP), Montpellier, France, pp: 36.

Loomis, W.D., 1974. Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. Methods Enzymol., 31: 528-544.

MoA., 2003. The gazette of India extraordinary. Ministry of Agriculture (MoA), New Delhi.

Mullis, K.B. and F.A. Faloona, 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Methods Enzymol., 155: 335-350.

Porebski, S., L.G. Bailey and B.R. Baum, 1997. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Mol. Biol. Rep., 15: 8-15.

Sambrook, J. and D.W. Russell, 2001. Molecular Cloning: A Laboratory Manual. 3rd Edn., Cold Spring Harbor Laboratory Press, New York, USA.

Souza, H.A.V., L.A.C. Muller, R.L. Brandao and M.B. Lovato, 2012. Isolation of high quality and polysaccharide-free DNA from leaves of *Dimorphandra mollis* (Leguminosae), a tree from the Brazilian Cerrado. Genet. Mol. Res., 11: 756-764.

Su, H.J., L.Y. Tsao, M.L. Wu and T.H. Hung, 2003. Biological and molecular categorization of strains of *Banana bunchy top virus*. J. Phytopathol., 151: 290-296.

Thompson, J.D., D.G. Higgins and T.J. Gibson, 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res., 22: 4673-4680.

Weising, K., H. Nybom, M. Pfenninger, K. Wolff and G. Kahl, 2005. DNA Fingerprinting in Plants: Principles, Methods and Applications. 2nd Edn., CRS Press, New York, ISBN: 9781420040043, Pages: 472.

Wuyts, N., D. De Waele and R. Swennen, 2006. Extraction and partial characterization of polyphenol oxidase from banana (*Musa acuminata* Grande naine) roots. Plant Physiol. Biochem., 44: 308-314.