



OPTIMIZATION OF MULTIPLEX-PCR FOR SIMULTANEOUS DETECTION OF VIRUSES INFECTING BANANA IN ANDHRA PRADESH

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ABSTRACT

Banana is an economically important major fruit crop in many tropical and subtropical countries. Its fruit provides remarkable nutritional values to the consumers. Banana is now under serious threat by various diseases in which viral diseases were predominant and account for significant yield loss. India ranked first in the world banana production. In India, viruses viz., Banana bract mosaic virus (BBRMV), Banana streak virus (BSV), Cucumber mosaic virus (CMV) and Banana bunchy top virus (BBTV) were commonly identified in banana and causes bract mosaic, leaf streak disease, infectious chlorosis and bunchy top diseases, respectively. A rapid and accurate disease diagnosis enables successful management of pathogens. In the present study a multiplex-PCR was optimized to detect BBRMV, BSV, and CMV simultaneously in banana. The amplified genomic regions for BBRMV, BSV and CMV were coat protein (389 bp), RT/RNaseH (597 bp) and movement protein (831 bp) respectively. The present developed multiplex-PCR technique is reliable, reproducible and quickness in detection of BBRMV, BSV and CMV in banana.

KEY WORDS

Banana bract mosaic virus, Banana streak virus, Cucumber mosaic virus, multiplex-PCR,

INTRODUCTION

Banana is one of the widely cultivating fruit crops in India. It is a vegetative propagated largest perennial herb, relatively inexpensive and every part of plant is made use of in various ways. According to the FAOSTAT (2016), India ranked first in the world banana production (29.1 million tonnes) [1]. Cultivated banana and plantain originated mainly from two wild species i.e., *M. acuminata* (AA genome) and *M. balbisiana* (BB genome) resulting in a series of diploid, triploid and tetraploid genomes (AA, AB, AAA, AAB, ABB, AABB, AAAB, ABBB), which form three types of food dessert, cooking (including plantain), and beverage [2]. Banana is susceptible to many viral diseases and account for severe yield loss worldwide. So far, 18 viruses viz., "Banana bunchy top virus (BBTV, a Babuvirus), Banana streak Mysore virus (BSMYV, a Badnavirus), Banana

streak IM virus (BSIMV, a Badnavirus), Banana streak UA virus (BSUAV, a Badnavirus), Banana streak UI virus (BSUIV, a Badnavirus), Banana streak UL virus (BSULV, a Badnavirus), Banana streak UM virus (BSUMV, a Badnavirus), Banana streak VN virus (BSVNV, a Badnavirus), Banana streak Gold finger virus (BSGFV, a Badnavirus), Banana streak Obino l'Ewai virus (BSOLV, a Badnavirus), Banana bract mosaic virus (BBRMV, a Potyvirus), Banana mild mosaic virus (BanMMV, Betaflexiviridae), Banana virus X (BVX, Betaflexiviridae), Cucumber mosaic virus (CMV, a Cucumovirus), Abaca mosaic virus [AbaMV, a distinct strain of Sugarcane mosaic virus (SCMV) designated as SCMV-Ab, a Potyvirus], Abaca bunchy top virus (ABTV, Babuvirus), Tobacco mosaic virus (TMV, a Tobamovirus) and Banana die-back virus (BDBV, a probable Nepovirus)" were reported to infect banana worldwide

[3]. In India, BSV, BBrMV, CMV and BBTV are the major viruses threatening banana cultivation and international exchange of germplasm. The first three viruses were distributed all over the India and account for 18 to 50% yield loss in banana [4, 5, 6]. Disease management is the best option to control these viruses. This can be achieved by selecting the resistant cultivars and early detection of pathogens. In case of banana, so far no resistant cultivars available for these viruses, hence, the alternative choice is to adopt a highly reliable and reproducible technique to diagnose these viruses at an early stage in banana. PCR is one of the best diagnostic methods in accurate detect of viruses, however, each virus detection separately is time consuming and expensive. Hence, in the present study we have optimized multiplex-PCR for simultaneous detection of BBrMV, BSV and CMV in banana plants. BSV is a double stranded DNA virus belongs to the genus *Badnavirus* (family, *Caulimoviridae*) causes banana streak disease" (BSD) in banana. Similarly, BBrMV (genus, *Potyvirus*; family, *Potyviridae*) and CMV (genus, *Cucumovirus*; family, *Bromoviridae*) are single stranded RNA viruses causes bract mosaic and infectious chlorosis in banana, respectively. Sometimes virus infected banana may not express symptoms and sometimes more than two viruses together express similar symptoms that lead to more confusion for their correct detection. The present multiplex-PCR is a highly sensitive, specific and reliable in detection of multiple viruses at a time in banana.

MATERIALS AND METHODS

Plant material

PCR confirmed BSV, BBrMV and CMV infected banana plant materials (leaf samples) were used in this study to optimize the multiplex-PCR technique.

Total DNA isolation from BSV infected banana

Total DNA was isolated as described by Dellaporta et al., [7] method with minor modifications. A total of 100 mg banana leaf sample was macerated in a sterile mortar and pestle by adding liquid nitrogen. The resulted powder was carefully transferred into a 2 ml of eppendorf tube and to it added 1 ml of pre-heated (95°C) extraction buffer (Tris 99.67mM, EDTA 49.97mM, NaCl 49.62mM and 70µl of 2-mercaptoethanol) and 33 µl of 20% SDS and again incubated at 95°C for 10 min with occasional agitation by vortexing. After incubation the tubes were placed on ice for 2 min and centrifuged at 12,000 rpm for 10 min. RNase (100 µg/ml) treatment

was made to the resulted supernatant (1:1, v/v). Then the sample was extracted with phenol-chloroform - isoamyl alcohol (25:24:1, v/v/v) and centrifuged at 10,000 rpm for 5 min. Supernatant was carefully collected into a new 1.5 ml eppendorf tube and to it added 0.6 volumes of ice-cold isopropanol and centrifuged at 14,000 rpm for 10 min. The resulted pellet was washed twice with 70% alcohol and dissolved in sterile double distilled water (50 µl).

Total RNA isolation from BBrMV and CMV infected banana

The virus (BBrMV or CMV) infected banana samples (leaf/pseudostem) and the corresponding healthy samples were collected and washed properly with distilled water, followed by DEPC treated distilled water and air dried. Approximately 100 mg of leaf material was used for isolation of total RNA by following the Qiagen total RNA isolation kit method.

cDNA synthesis by RT-PCR

A 20 µl reaction was setup for the first strand cDNA synthesis, 1 µg of template RNA and 0.5 µg of Oligo dT/sequence specific reverse primers were taken into an RNase-free 0.2 ml eppendorf tubes and incubated the mix at 70°C for 5 min and chilled on ice. To the mix, the following reagents i.e., 5x reaction buffer, 10 mM dNTPs mix and ribonuclease inhibitor were added as described in the manual (Thermo Scientific), mixed well with pipette and incubated for 5 min at 37°C. Finally, 200 U of M-MuLV reverse transcriptase (Thermo Scientific) was added to the mix and incubated for 1 h at 42°C. The reaction was stopped by heating the mixture for 10 min at 70°C.

Primer design and synthesis for multiplex-PCR

Based on the nucleotide sequences of the viruses, primers were designed using the software's Bio-edit, Oligo6, NCBI-BLAST and primer 3 to detect BSV, CMV and BBrMV simultaneous by multiplex-PCR. These primers were specific to amplify RT/RNaseH, movement protein (MP) and coat protein (CP) gene regions of BSV, CMV and BBrMV, respectively (Table 1). The designed primers were synthesized commercially from Alpha DNA, Germany and MWG Bangalore, India. A 100 pico moles (pm) primer stock was prepared with sterile DEPC treated double distilled water and the working concentration (10 pm) was made from stock, aliquoted and stored at -20°C.

Multiplex-PCR for simultaneous detection of BBrMV, BSV and CMV

For simultaneous detection of BBrMV, BSV and CMV, a 50 µl reaction was set. Initially, total RNA for BBrMV and CMV and total DNA for BSV were isolated from infected *Banana* as described previously. First strand cDNA synthesis for BBrMV and CMV was carried out in a single vial by adding Oligo-dT and sequence specific primers, respectively. Later, approximately 2 to 3 µg of cDNA and total DNA was taken into a 200 µl pcr tube and to it added ingredients i.e., 5X reaction buffer (5µl), 25 mM MgCl₂ (4 µl), 10mM dNTPs mix(1µl), forward and reverse primers of BSV, CMV and BBrMV (10pm) each (2 µl), Taq polymerase (2 U, Thermo Scientific) and made up to 50 µl with sterile double distilled water. The cyclic conditions for multiplex-PCR were, denaturation at 94°C/1min, annealing at 50°C/1 min and extension at 72°C/1 min with a total of 35 cycles and a final extension time at 72°C/10 min. The PCR products were analyzed by 1% agarose gel electrophoresis (fig1)

RESULTS AND DISCUSSION

The fruit banana is relatively inexpensive and a good source of several essential micronutrients, hence, their consumption is dramatically increased worldwide. The world banana and plantain production was 148 million tonnes in which India (29.1 million tonnes) and China (13.1 million tonnes) have collectively occupied 28% of global banana production [1]. However, an important amount of banana production is lost every year due to multiple diseases. There were about 18 viruses have been reported in banana responsible for significant yield loss globally. In India, yield and quality of banana was highly influenced by three major viruses including BSV, BBrMV and CMV. Early detection of these viruses minimizes the crop damage. ELISA is one of the ideal diagnostic techniques in virus detection in host plants, however, it remains depends on the quality of the antibodies available and the nature of host plant. Plants like banana and yam have high levels of polyphenols and polysaccharides, and hence their effects reflect on the specificity and sensitivity of ELISA in detection of viruses. PCR is one technique has high specificity and accuracy in virus detection. However, detection of

individual viruses separately by PCR is time-consuming and expensive. Recently the technique multiplex-PCR has gained momentum in plant virology. More than two viruses can be detected paralally in a single PCR vial, which save time and cost of the experiment. Multiplex-PCR was developed for detection of several plant viruses. For example, multiplex-RT-PCR has been developed to detect five potato viruses viz., PVA, PVS, PVX, PVY and PLRV simultaneously in a single PCR tube [8]. Similarly, a duplex-RT-PCR has been reported for detection of PLRV and PVY [9]. In the present study a multiplex-PCR was developed to detect BBrMV, BSV and CMV In infected banana. The selected genome regions for the amplification of BBrMV, BSV and CMV were CP (389 bp), RT/RNaseH (597 bp) and MP (831 bp) respectively. Care was taken to design primers for their amplification. For BBrMV and CMV amplification, total RNA was isolated by Qiagen RNAeasy isolation kit and synthesized first strand cDNA simultaneously in a single PCR vial by adding Oligo- dT and sequence specific reverse primer, respectively. To this cDNA reaction mix, total DNA isolated from BSV suspected *Banana* was added and carried out the experiment. The expected genome products for three viruses were amplified (Fig.1). The success of the technique also depends on the concentrations of the template. For example, during the optimization of the technique, it was found that addition of more genomic DNA template hampered the amplification of gene product of BBrMV. A multiplex-immunocapture PCR (M-IC-PCR) was developed for the simultaneous detection of three viruses viz., BBTv, BBrMV and CMV from crude sap extracts of banana and plantain (*Musa* spp.) in Australia [10]. Bariana et al., [11] have developed for simultaneous detection of seed-borne legume viruses like AMV, MYMV, CYVV, CMV and SCMoV that are great concern to legume germplasm banks. In this study, the developed multiplex-PCR could be useful for parallel detection of BSV, BBrMV and CMV in symsptomatic or asymptomatic banana. This test can be used for screening of germplasm of banana and for selection of mother plants for mass multiplication by tissue culture technology.

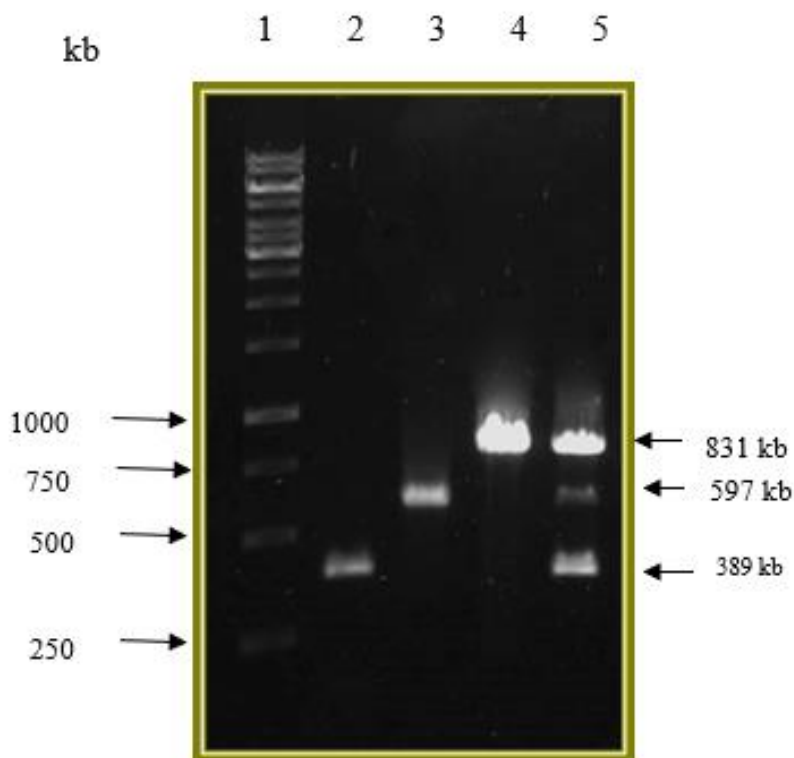


Fig 1. Parallel detection of BBrMV, CMV and BSV in banana, by multiplex-PCR. Lane 1-DNA marker; lane 2- BBrMV, lane 3- BSV, lane 4- CMV and lane 5-BBrMV (389 bp),BSV(597 bp) and CMV (831 bp).

Table 1. Primers for multiplex-PCR detection of BBrMV, BSV and CMV

Virus (Amplified gene region)	Primer	
	Forward	Reverse
BBrMV (Part of CP gene)	5'GTGAGCAATTAGTTTACCAG3'	5'GTCAGAATTCTTATTCA TGTTTCATC3'
BSV (RT/RNaseH)	5'CTNTAYGARTGGYTG ATGCCNTTYGG3'	5'TCCAYTTRCANAYNSCN CCCCANCC3'
CMV (MP)	5'CCAAGGTACAGTAGGAC3'	5'AAAGACCGTAACCACCTG3'

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