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DETECTION OF BANANA BRACT MOSAIC POTYVIRUS BY IMMUNO CAPTURE POLYMERASE CHAIN REACTION (IC-PCR)

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ABSTRACT

The characteristic bract mosaic symptom caused by *Banana bract mosaic potyvirus* (BBrMV) is observed only during flowering on the flower bract and due to this reason, it is very often not recognised during young stages of growth. The disease is widespread and its early identification is important to prevent economic losses and further spread. Banana bract mosaic virus was purified from infected *embul* banana plant leaves. A polyclonal xanliserum was produced to a Sri Lankan strain of BBrMV. One step reverse transcriptase (RT) PCR and immunocapture RT-PCR were developed to detect BBrMV. Primers were designed within the coat protein coding region and gave an amplification product of cc. 320 bp. IC-RTPCR was more labour efficient and less costly than one step RT-PCR.

KEYWORDS: Banana bract mosaic potyvirus, RT-PCR, IC-RTPCR

INTRODUCTION

Banana (*Musa* spp.) is grown both as a staple fruit as well as a cash crop mainly for the local market. Constraints for its cultivation at present are diseases caused by a number of viruses including banana bunchy top virus (BBTV), cucumber mosaic virus (CMV), banana streak virus (BSV), and banana bract mosaic virus (BBrMV). Banana bract mosaic virus was first observed in the Philippines in 1979 (Magnaye and Espino, 1990) and subsequently yield losses up to 40 % have been recorded (Roperos and Magnaye, 1991). In Sri Lanka Thomas et al first confirmed its presence in 1997. The disease has at present been reported from 3 main agro-ecological zones of Sri Lanka.

The characteristic symptoms of this disease are the green or reddish brown broad spindle shaped streaks along the petioles and pseudostem (figure 1)[1], dark brown streaks on the bracts of the inflorescences. Spindle shaped chlorotic lesions are also sometimes evident on the leaves. The disease has been so named after its characteristic mosaic symptoms on the flower bract (figure 2). This however is evident only during the flowering stage. (Magnaye and Espino, 1990).

BBrMV is a member of the Potyvirus genus in the *Potyviridae* (Bateson and Dale, 1995; Thomas *et al.*, 1997). It has been transmitted experimentally to healthy banana plants by aphid species *Rhopalosiphum maidis* and *Aphis gossypii* (Magnaye

[1] Figures are provided in the plate at end of the paper.

and Espino, 1990) in a non-persistent manner. Flexuous filamentous virus like particles have been observed in sap extracts (Espino *et al.*, 1990). No other hosts of the disease have been reported and the virus is not mechanically transmitted (Diekmann and Putter, 1991; Munez, 1992). Banana bract mosaic virus poses a considerable quarantine risk due to its ability to spread through vegetative plant parts and by aphids. Therefore a reliable diagnostic assay for its detection is required.

MATERIALS AND METHODS

Embul banana plants with symptoms of banana bract mosaic disease were collected from Angunukolapelesa, Homagama and Bombuwela. These were maintained in an aphid-protected greenhouse at the Regional Agricultural Research Centre, Bombuwela. Samples from some of these plants were sent to Dr. John Thomas at the Department of Primary Industries, Queensland, Australia and to Professor Ben Lockhart at University of Minnesota, USA to confirm the presence of Banana bract mosaic virus in those. Those were further tested in Plant Virus Indexing Centre, Gabadawatte, laboratories by indirect ELISA using an antiserum received from B.Rodoni of the Queensland University of Technology, Queensland, Australia.

Antiserum Production

Samples from those plants infected with the BBrMV only, without the presence of other viruses were selected for the extraction and purification of the virus. BBrMV was purified according to the method described by Hammond and Lawson (1988). This method involves extraction of the virus using 0.5 M K_2HPO_4 / KH_2PO_4, pH 8.4 + 0.5 % Na_2SO_3, buffer, clarification by Triton X-100, concentration by polythene glycol and isopycnic centrifugation in caesium sulphate. Specific virus band was removed manually by using a hypodermic syringe.

Antiserum to BBrMV was prepared in rabbits using schedule of one intraveinous injection (0.5 mg virus in 0.85% saline) followed by five intramuscular injections (1 mg virus emulsified with an equal volume of Freunds incomplete adjuvant (Difco Ltd.,) given at weekly intervals.

Polymerase chain reaction (PCR) Primers

Two primer pairs designated to the coat protein coding region were used. Bract 1:5' GAC ATC ACC AAA TTT GAA TGC CAC ATGG 3' and Bract 2:5'

CCA TTA TCA CTC GAT CAA TAC CTC ACAG 3' described by Rodney *et al.*, (1999). An additional primer pair F_1 - AAC GCT CAG CCT ACT TTT CG and Primer R - CAT ATC ACG CTT CAC ATC TTC A were designed using BBrMV sequence (Gene bank accession AJ 071586). Additionally an Oligo dT primer (5'-P (dT_{12}) – dx – dy – OH –3-) was used for cDNA synthesis in IC-PCR,

The use of Oligo dT primers for the synthesis of first strand cDNA copy of viral genomic RNA's having 3' poly A. tail has been practised as a standard molecular biological technique for the detection of viruses such as potyvirus spp.

Nucleic Acid Extraction

A nucleic acid extraction protocol was provided by Prof. H.J.Su (National Taiwan University, 1999). Leaf sample of 0.5g was extracted with 3 ml of DNA extraction buffer (100 mM Tris HCl, 100 mM EDTA, 250 mM NaCl pH 8.0) + 1 % Sarcosyl (N-Lauroyl sarcosine) (2.7 ml of extraction buffer and 0.3 ml of 10 % Sarkosyl). Tissue powder suspension was then stirred and transferred to a 1.5 ml eppendorf tube and incubated at 55 ° C for 1 hour in a water bath. Tubes were then centrifuged at 6000 rpm for 5 minutes. After centrifugation, 800 µl of supernatant was collected. This was then incubated for 10 minutes at 65° C after addition of 100 µl of 5 M NaCl and 100 µl of CTAB (Hexa-decryl trimethyl ammonium bromide) / NaCl (10% CTAB in 0.7 M NaCl). 600 µl of chloroform: isoamyl alcohol (24:1) was then added and mixed thoroughly and spun at 11,000 rpm for 10 minutes. Supernatant was collected and 600 µl of Phenol / chloroform / iso amyl alcohol (25:24:1) was added and mixed thoroughly.

Tubes were spun at 11,000 rpm for 10 minutes. Supernatant was collected and 360 µl of isopropanol was added to precipitate the nucleic acid. Tubes were incubated at 20° C for overnight and spun at 12,000 rpm for 15 minutes. Pellets were washed with 70 % ethanol to remove CTAB residues, then briefly dried and resuspended in 100 µl of TE buffer, 3 µl of this extract was used for reverse transcriptase reaction.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

The PCR reaction mixture (25 µl) contained 5 x RT buffer – 5 µl, 100 mM DTT – 1.25 µl , Reverse transcriptase (Gibco BRL – Life Technologies, 200 units / µl) 0.25 µl, DNTPS (10 mM) 2 µl, Primers F_1 & R mixed 50 mg / µl each 2 µl or

Bract 1 & Bract 2 (34 pmol each) 2 µl , RNA template 3 µl and 11.5 µl of DD water. The amplification protocol involved, one cycle of 50° C for 35 min, one cycle of 94° C for 2 min; 35 cycles of 94° C for 30 sec, 50° C for 30 sec, 68° C for 45 sec and one cycle of 68° C for 7 min and 4° C soaking.

Immuno Capture PCR (IC-PCR)

Thin walled micro-centrifuge tubes were coated with BBrMV specific antiserum produced locally, diluted at 1:200 in 0.05M Sodium carbonate buffer, pH 9.6 and incubated for 3 hours at room temperature. After incubation tubes were washed with Phosphate buffered saline (PBS-T) {01M phosphate (Na_2PO_4 / KH_2PO_4) + .15M sodium chloride + .003 M potassium chloride pH 7.4} and 0.5 ml/L Tween 20 (Sigma Ltd.) 3 times, each for 3 minutes.

Leaf samples were extracted at the rate of 0.5 g of leaf tissue in 2.5 ml of virus extraction buffer (0.2M, K phosphate pH 7.0, 15 mM EDTA, 2% PEG, 0.5% Na_2SO_3). Fifty micro litres of extracts were then added and the tubes were incubated overnight at 5 ° C. After incubation the standard washing step was followed using PBS-T buffer. Tubes were dried at room temperature and centrifuged at 3000 rpm for 3 minutes. An Oligo dT primer (0.5 g / µl) 1 µl and 23 µl of distilled water was added to the immuno-capture tubes.

Tubes were then heated to 80° C for 10 minutes in a water bath, chilled on ice and spun down briefly. Following mixture was then added: 5 x 1st strand buffer 8 µl, 0.1M DTT 4 µl, Reverse transcriptase (M-MLV) 1 µl, (25 units/µl), dNTPS (10 mM) 2 µl and RNase inhibitor (5 units / µl) 1 µl. Tubes were then incubated at 42° C for 1 hour, 75° C for 15 minutes and chilled on ice or at –20 ° C. Three micro litres of cDNA template was then used for PCR amplification. PCR mixture used was 10 x PCR buffer 2.5 µl, $MgCl_2$ (25 mM) 2.0 µl, dNTPS (10 mM) 1.0 µl, Primer (mixed) 2 µl (either Bract 1 / 2 or F_1 / R conc. as RT-PCR), Taq (2 units / µl) 0.25 µl, cDNA template 3 µl and distilled water 14.25 µl. Thermal cycler parameters were one cycle of 94° C for 1 min followed by 33 cycles of 94° C for 30 sec, 56° C for 1 min, 72° C for 1 min and one cycle of 72° C for 3 min. Tubes were soaked at 4° C for 2 hours before electrophoresis.

Analysis of PCR products

Amplified PCR products were analysed by electrophoresis at 70 V for 90 minutes in 1% agarose gel in TAE buffer (40 mM Tris HCl, 5 mM Sodium acetate, and 1 mM EDTA pH 7.7 per litre) in the presence of .05 µg / ml ethidium bromide and photographed under ultra violet light. DNA molecular marker 1X (HT Biotechnology Ltd.) was used as size marker.

RESULTS AND DISCUSSION

Polyclonal antiserum

For serological investigations and IC-PCR detection the final bleed of antiserum was used to maintain its specificity. Efficacy of locally produced antiserum was compared with the commercially produced BBrMV antiserum by Agdia commercial kit (results are not presented in this paper). The comparison showed that locally produced antiserum has the ability to detect bananas infected with BBrMV.

BBrMV amplification by IC-PCR /RT-PCR method

When Bract $_1$ and Bract $_2$ primers were used for RT-PCR and IC-PCR it was not possible to amplify the relevant genomic portion for the infected samples collected from different locations. However the primer pair F_1 / R successfully amplified about 324 bp product both in RT-PCR (one step method) and IC-PCR method (figure 3). In this method, polyclonal antiserum used was the same as used for serological tests. This will reduce the antiserum costs.

The polymerase chain reaction technique for the diagnosis of plant viruses is aided by a rapid, simple, and reliable method for viral nucleic acid detection. The crude sap extracts used in IC-PCR meet this requirement and eliminate the need for hazardous chemicals used in the more time-consuming total nucleic acid extraction protocols. Other added advantage in IC-PCR is it only traps virus particles and not genomic DNA from *Musa.*

In the present series of investigations two primer pairs were compared in PCR to detect the local isolate of BBrMV. Only the primer pair F_1 / R was successful with the local isolates of BBrMV, while the results were negative when Bract $_1$ and Bract $_2$ primer pair was used. Sharman *et al.* (2000) found that 2 isolates collected from India appear to lack a functional Bract$_2$ primer site and that it was

present in other Indian isolates (Personal communication with Ms.Cherie Gambly, QDPI, Queensland, Australia). Therefore the negative results in the present investigations suggest that Sri Lankan isolates of BBrMV lack the $Bract_2$ primer site.

IC-PCR method was utilized for indexing local samples as well as those received from other countries. With high priority on Agricultural development emphasis is on good quality planting material, which could result in international exchange of promising cultivars. This should be encouraged only with strict quarantine practises supported by sensitive virus detection technologies.

CONCLUSIONS

Sri Lankan made polyclonal antiserum for banana bract mosaic virus is useful in virus detection by serological and molecular based methods. F_1 / R primer pair was found as a suitable primer for the amplification of banana bract mosaic virus in Sri Lanka. Furthermore Immuno-capture polymerase chain reaction is a more convenient method than one step RT-PCR method. Therefore routine indexing of BBrMV can be done by use of IC-PCR methodology.

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REFERENCES

Bateson, M.F. and J.L.Dale, 1995. Banana bract mosaic virus: Characterisation using potyvirus specific degenerate primers. Arch. Virol. 140: 515-527.

Diekmann, M. and C.A.J.Putter. 1996. FAO/IPGRI Technical guidelines for the safe movement of germplasm. No. 15, Musa. 2nd Edition. Food and Agricultural Organisation of the United Nations. Rome/International Plant Genetic Resources Institute, Rome.

Espino, T.N., S.B. Exconde, F.B. Zipogan, and R.R.C.Espino, 1990. Banana bract mosaic, a new disease of banana: II. Isolation and purification for monoclonal antibody production. Phillip. Agric. 73: 61-68.

Hammond, J. and R.H. Lawson, 1988. An improved purification procedure for preparing potyviruses and cytoplasmic inclusions from the same tissue. J. of Virol Methods. 20: 203-217.

Magnaye, L.V. and R.R.C.Espino, 1990. Banana bract mosaic, a new disease of banana. 1. Symptomatology. Phillip. Agric. 73: 55-59.

Munez, A.R. 1992. Symptomatology - transmission and purification of banana bract mosaic virus (BBMV) in *Giant Cavendish* banana. Master of Science thesis, University of Philippines, Los Banos. 57p.

Rodoni, B.C., J.S. Ahlawat, A.Varma , J.L.Dale, and R.M.Harding 1999. The identification and characterization of banana bract mosaic virus In Southern India Plant Disease 81: 669

Roperos, N.I. and L.V.Magnaye, 1991. Status of banana diseases in the Philippines In Banana diseases in Asia and the Pacific. Pp 52-66.

Su, H.J. 1999. Development and Application of molecular diagnostic probes for detection, characterization and management of banana viruses. INIBAP-ASPNET (RAC) Meeting Guangzhou, China. November 2-5, 1999.

Thomas, J.E., A.W.Geering, C.F.Gambley, A.F.Kess;ing and M.White, 1997. Purification, Properties and Diagnosis of Banana Bract Mosaic Potyvirus and its distinction from Abaca Mosaic Potyvirus". Phytopathology. 87: 698-704.

Figure 1. Pseudostem of banana infected with BBrMV showing unusual reddish colouration

Figure 2. Characteristic mosaic symptom in flower brac

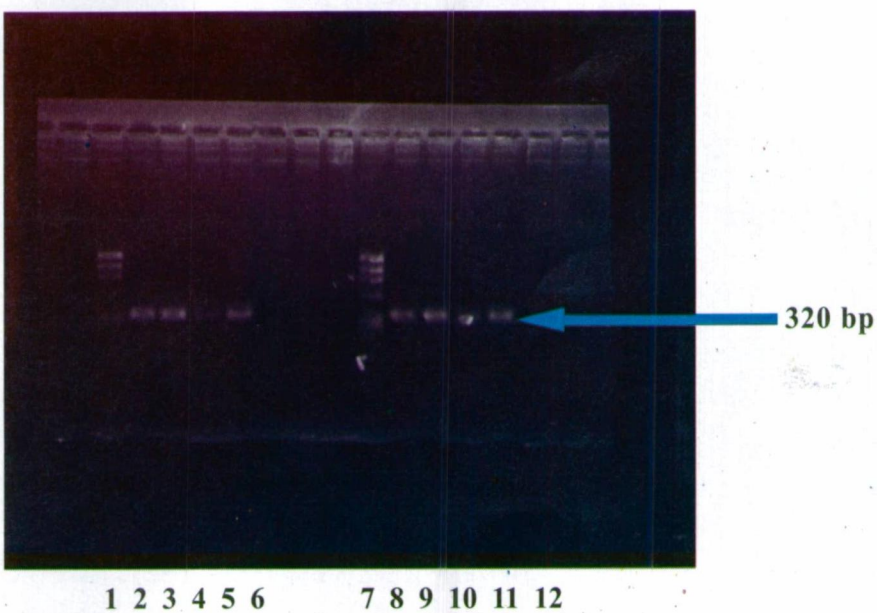

Figure 3. Detection of BBrMV by one step RT-PCR and IC-PCR using primer pairs F_1/R.

Lane 1 & 7	DNA molecular size marker,
Lane 6 -	Apparently healthy sample
Lanes 2-5	Amplified product from RT-PCR method (different samples)
Lanes 8-11	Amplified product from IC-PCR method
Lane 12	Apparently healthy sample