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Comparison of Detection Methods for Banana Bract Mosaic Virus in Banana (*Musa* sp.)

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Abstract: Banana bract mosaic virus (BBrMV) locally known as Kokkan causes serious threat to banana production and it spreads at a high rate in almost all the banana varieties cultivated in Kerala. Serological techniques i.e. chloroplast agglutination, microprecipitin test, agar gel double diffusion test, Direct Antigen Coating-Enzyme Linked Immunosorbant Assay (DAC-ELISA) and Dot Immunobinding Assay (DIBA) were compared to evaluate the performance of the diagnosis of the virus. Each method was carried out using extract of infected tissues and antisetrum developed against the virus. ELISA and DIBA were found to be more sensitive compared to the other methods tested. DAC-ELISA was found to be more economical method for the detection of (BBrMV).

Key words: Agar gel double diffusion · BbrMV · chloroplast agglutination · DIBA · ELISA · microprecipitin test

INTRODUCTION

Banana and plantains grown well in India with great cultural and socio-economic significance. Banana agriculture is subject to many natural calamities, but diseases constitute a major problem. Virus diseases are serious, as insect vectors are abundant and there are many alternate hosts. The virus disease causing most concern is banana bract mosaic, which affects many cultivars and is spreading rapidly throughout the India and Philippines [1]. The virus that have been characterized, BBrMV, is found in plants of all age groups and primarily transmitted through infected suckers and the secondary spread is by the viruliferous aphid, *Pentalonia nigronervosa* Coq. [2]. Unlike bacterial and fungal diseases, viral diseases are difficult to manage because of complex disease cycle, efficient system of transmission and non-availability of viricides. Since the main mode of transmission of BBrMV, rather than aphids, is through infected suckers the effective way for the management of the disease is the use of disease free suckers. Therefore indexing of stock plants for elimination of virus infected plants prior to planting is essential and requires the application of effective and reliable detection methods.

Biological method of detection as inoculation of indicator plants was found unsuccessful for BBrMV. Because of the sensitivity and relative ease recently serology based techniques are commonly employed for disease diagnosis [3]. The present work conducted with the evaluation of various serological techniques for the detection of BBrMV.

MATERIALS AND METHODS

Various serological techniques as chloroplast agglutination, microprecipitin test, agar gel double diffusion, ELISA and DIBA were performed and compared to detect BBrMV in infected banana suckers.

BbrMV was partially purified [4] from infected leaf lamina and was injected at weekly intervals to New Zealand white rabbit intra muscularly. Blood was taken 10 days after the fifth injection and serum separation was used as antiserum. Meristem cultured banana plants (Nendran) after virus indexing were used for taking healthy samples.

The above mentioned techniques were used to detect BBrMV in infected samples.

Chloroplast agglutination test: Crude sap from the leaf samples were squeezed through a muslin cloth and mixed with antiserum, developed against the virus and the reaction was observed under stereo binocular microscope.

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Microprecipitin test: Infected samples were extracted in 0.1 M phosphate buffer (pH 7.0).10 µl of the clarified extract was mixed with equal quantity of the developed antiserum. The mixture was incubated at 25°C in a humid chamber for 30 minutes and examined under a microscope.

Agar gel double diffusion test: The test was carried out as the protocol suggested by Ouchterlony [5] with slight modification. The test was performed on microscopic slides, pre-coated with 0.5% formvar in chloroform. The slides were over layered with 2.5 ml of 0.75% molten agar in 0.05 M Phosphate Buffer Saline (PBS) pH 7.4 containing 0.22% sodium azide as preservative/biocide. Blocks of agar was removed with a cork borer of 4 mm diameter to give four member well pattern in equidistant position and at an angle of 40° to central well. Extracts of the tender leaves ground in PBS (1:1 w/v) were used as test antigens. The central well was loaded with 8 µl of cross absorbed BBrMV antiserum. Same quantity of BBrMV infected plant sap was loaded in the upper wells whereas lower wells received healthy plant sap. The slides were incubated in a humid chamber for 48 h for the antigen antiserum interaction.

Slides were immersed in 0.85% (w/v) aqueous NaCl for 12-24 h to remove unreacted proteins/antigen and antibody. The slides were repeatedly immersed in distilled water for about 3-4 h to remove the traces of salts and dried in a bell jar for 2-3 days at room temperature. The air dried slides were stained with Amido Black 10 B stain, 0.6% dissolved in methanol: glacial acetic acid: distilled water (45: 10: 45 ml).There after the slides destained in the same mixture without the stain (Wieme, 1959). Contact prints were taken by placing the gel side over the emulsion side of the photographic paper.

DAC-ELISA: The assay was conducted following Clark and Adams method [6] with slight modifications in the procedure of antigen extraction from the standard procedures. Instead of purified virus crude sap from the plants were taken as antigen for the analysis.

Since partially purified virus was used for the production of antiserum it was cross absorbed for 2 h at 37°C with an equal volume of healthy banana extract to remove the antibodies against host protein. Cross absorbed antiserum was centrifuged at 1000 rpm for 5 minutes and the supernatant was used for DAC-ELISA.

Titre of the antiserum was determined by DAC-ELISA. Different dilutions of antiserum viz., 1: 128, 1: 256, 1: 512, 1: 1024 were tried. Field sample (leaf, pseudostem and bract) were used as antigen whereas meristem cultured, virus indexed banana plants served as control.

Samples extracted 1:5 (w/v) in carbonate buffer were initially loaded to be tested into Microtitre plates (100 µl well^{-1}). Then incubated for 2 h at 37°C. The plates were then washed three times with PBS-T and loaded with 100 µl of 1% BSA for blocking. Wells were loaded with 100 µl of developed antiserum (1:512 dilution) in PBS-TPO. Plates left overnight at 4°C then washed and was loaded with 100 µl of ALP conjugated 1 gG (1 mg ml^{-1}) in PBS-TPO. Finally 100 µl Para Nitro Phenyl Phosphate (pNPP) loaded in diethanolmine (1:1000) was loaded into the well. After incubation for 2 h at 37°C and washing the absorbance reaction was read after 15-20 minute incubation at room temperature using an ELISA reader (ECIL MS5608) at 405 nm.

DIBA: Nitrocellulose membrane (NCM) was pre-soaked in Tris buffer saline (TBS) and air dried. NCM was divided into squares of 1 cm using a lead pencil. Sample extracted in TBS 50 mM DIECA (1:10 w/v) was clarified by mixing with chloroform (1: 0.5 v/v) and centrifuging at 10,000 rpm for 10 minutes. 10 µl of the upper aqueous layer was spotted in the centre of the square on NCM. NCM was blocked with 5% SDM in TBS buffer for one hour at room temperature by shaking. The membrane was incubated overnight with antiserum (1:512) in TBS-SDM after a rinse in TBS. After washing NCM, it was incubated with goat anti-rabbit ALP conjugated IgG (Sigma A3687) in TBS SDM (1 mg ml^{-1}) for one hour at room temperature. Then after, the membrane washed and dried between filter paper s. For colorimetric detection, the membrane was incubated in a freshly prepared substrate buffer containing BCIP (0.175 mg ml^{-1}) and NBT (0.33 mg ml^{-1}). After the colour development NCM was rinsed in Tris EDTA solution for 10 minutes for fix the colour, then air dried between Whatman filter paper sheets and stored.

A dilution series of BBrMV infected sample extract in healthy banana extract (1:10 v/v) were carried out to check the efficacy of the detection system ELISA and DIBA. Dilutions tried for sensitivity tests were 1: 100, 1:250, 1:500 and 1:1000. Simultaneous assays were made using other precipitin method.

RESULTS AND DISCUSSION

In the present study various methods for viruses detection were evaluated.

The obtained results revealed that the infected samples when examined in chloroplast agglutination test gave a dense precipitate whereas that was absent for healthy sample. Similar recorded results by Estelitta [7] confirmed the efficacy of this test for the detection of virus diseases of banana.

Micro precipitin test also gave positive result when infected samples were used which was indicated by the development of a white precipitate. Parvin and Khan [8] successfully used micro precipitin test for the detection of poly virus in potato.

Agar gel double diffusion test also gave positive reaction for infected sample which was indicated by the development of well defined white precipitin bands (Plate 1). The double diffusion method that recorded to be reliable for the detection of potyvirus in potato by Basu and Giri [9] and for banana bunchy top virus in banana by Estelitta [7] than other precipitin test, due to its visibility.

Even though these methods were found to be effective in detection of the pathogen, the reaction sensitivity of these was lesser. Moreover, samples with low virus concentration (higher dilution of the BBrMV infected sample extract) did not give any precipitate and quantity of antiserum required for single reaction was also high. Therefore these tests were not economical when large number of samples has to be tested.

Titre of the antiserum determined through DAC-ELISA was 1:512. The infected banana sample tested in DAC-ELISA positively reacted with the antiserum developed in the present experiment (Plate 2). Absorbance value read in ELISA reader was compared with that of healthy sample. In this experiment replicated samples having a mean absorbance value which is about two times that of healthy was considered infected. Result of the experiment showed that the concentration of the virus was more in infected bract (A 405 = 1.16) followed by infected pseudostem and leaf (A_{405} = 0.82). Healthy sample showed an absorbance of 0.41. In this regard, Geering and Thomas [10] reported the efficacy of ELISA test for the detection of virus diseases of banana.

BbrMV infected samples gave positive reaction for DIBA and this could be detected by the development of purple coloured spots on nitrocellulose membrane (Plate 3).

Sensitivity test showed that higher dilution of BBrMV infected extract (1: 500 and 1: 1000) did not yield positive result for precipitation tests whereas ELISA and DIBA could detect the virus more efficiently.

ELISA and DIBA were found to be more sensitive test since they gave positive result for samples even which had low virus concentration. This might be due to larger affinity of antigen to polystyrene ELISA plate and NCM. The requirement of antiserum was less for ELISA and DIBA. According to Thomas *et al.* [11] minimum concentration of BBrMV detectable by ELISA was 16 ng ml^{-1}. Hsu and Lowson [12] reported that DIBA are at least as sensitive as multi wells ELISA for many viruses. Detection level for microprecipitin test is 500 ng ml^{-1} whereas that of ELISA is 10 ng ml^{-1} [13]. They also reported that compared to ELISA chloroplast agglutination test was 60% reliable. According to Parvin and Khan [8] the positive reaction of microprecipitin for detection of potyvirus was 75% whereas for ELISA its 78%. Selvarajan [14] reported that DIBA was most precise technique to detect BBrMV in infected field samples compared to other precipitation methods. According to Abad and Moyer [15] probability of non-specific reaction of antibodies and components of plant sap is higher in DIBA than in multi well ELISA. In this experiment, ELISA was found to be best suited and more cost effective (~4$ per sample) when large number of samples for the same virus is to be tested simultaneously.

CONCLUSIONS

Application of techniques described and compared here will be useful in routine, indexing programme of banana. However with its sensitivity and need for small quantities of infected tissue and antiserum and even a low virus concentration, ELISA and DIBA could be considered as efficient detection methods compared to other precipitin tests. Simplicity and applicability for large number of samples at economic rate make ELISA a more convenient and practical method of detection.

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