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Re-Evaluation of Yam Mosaic Virus (YMV) Detection Methods

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Abstract: Accurate and timely detection is vital for mitigation of tuber yield losses resulting from yam mosaic virus (YMV) infection on yam, a major food security crop in West Africa. The observation, from our previous studies, that the triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA), the most commonly used detection method for YMV, detected the virus in significantly less leaf samples than immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) necessitated a re-evaluation of YMV detection methods. In the present study, eighteen previously tested YMV positive leaf samples from Benin and Ghana were re-tested using TAS-ELISA, Protein A-sandwich (PAS) ELISA and IC-RT-PCR. Three sap dilutions, 1/10, 1/50 and 1/100, were tested for each sample. Both at 1/10 and 1/50 dilutions, PAS-ELISA and IC-RT-PCR detected YMV in 11 (61.1%) and 12 (66.7%) of the leaves respectively. Virus detection by PAS-ELISA reduced to 50% at 1/100 sap dilution and increased to 77.8% in IC-RT-PCR. YMV detection by TAS-ELISA varied between 38.9% and 16.7% at 1/10 and 1/100 dilutions respectively. These results indicate a deficiency in the use of TAS-ELISA as a sole YMV certification method since the detecting monoclonal antibody used in this assay may be strain specific. The use of PAS-ELISA at a 1/10 sap dilution is suggested for YMV detection where the facilities for molecular detection are unavailable.

Key words: *Yam mosaic virus* (YMV) · Detection sensitivity · TAS-ELISA · PAS-ELISA · IC-RT-PCR

INTRODUCTION

Yam mosaic virus (YMV), genus *potyvirus* is the most important virus infecting yam worldwide and has been reported to be widely distributed in all yam growing areas of the world [1-5]. Following its isolation and characterization from *Dioscorea cayenensis* in Côte d'Ivoire in 1979, extensive serological, molecular and epidemiological analysis of various isolates of YMV from across the world revealed that the virus is a single-stranded RNA potyvirus exhibiting high genetic diversity and consisting of more than one serotype [1, 2, 6]. The virus infects several species of yam including *D. rotundata, D.alata*, *D. cayenensis* and *D. trifida* producing various leaf symptoms of mosaic, mottle, shoe stringing and/or green vein banding.

Rapid detection and specific diagnosis of YMV is critical for disease management and for control of virus spread through quarantine certification. Several immunological and nucleic acid-based assays have been described for the specific diagnosis of YMV including triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA), dot-blot immunoassay (DBI), direct tissue blotting immunoassay (DTBI), immunosorbent electron microscopy (ISEM) and immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) [7, 8]. Of these methods, TAS-ELISA and IC-RT-PCR are more commonly used for specific diagnosis of YMV with TAS ELISA being the assay of choice for most researchers and quarantine agencies in Sub-Saharan Africa due to the expertise, equipment and reagent demand associated with the nucleic acid-based assay IC-RT-PCR [9-11]. Results from our previous studies revealed that IC-RT-PCR detected YMV in more leaf samples than TAS-ELISA [4, 5]. This result was particularly significant because virus trapping for both the TAS-ELISA and IC-RT-PCR in our previous studies was achieved using the same YMV polyclonal antibody,

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suggesting that the YMV monoclonal antibody used for virus detection in the TAS-ELISA may be responsible for the observed detection differences. The current study re-evaluates YMV detection using TAS-ELISA, IC-RT-PCR and protein-A sandwich (PAS) ELISA, an ELISA format that utilizes protein-A to enhance antibody sensitivity [12].

MATERIALS AND METHODS

Yam leaf samples from Benin (12) and Ghana (6) that tested positive for YMV in previous studies were used in this study [4, 5]. These samples had been sliced and rapidly desiccated in anhydrous calcium chloride on the same day they were collected in the field and have been in storage at 4°C at International Institute of Tropical Agriculture (IITA) Ibadan. Pre-tested YMV positive and YMV negative yam leaves at the IITA were used as positive and negative controls. Each of the leaf samples were tested in duplicate and at three different sap dilutions namely; 1:10 (w/v), 1:50 and 1:100. The YMV polyclonal and monoclonal antibodies used were from the IITA as previously described [4].

Enzyme-linked Immunosorbent Assay (ELISA): TAS-ELISA was carried out as previously described by Thottappilly *et al*. [13] with slight modifications. Test sap was prepared by grinding each leaf tissue in extraction buffer (PBS-T containing 2% polyvinyl pyrrolidone (PVP)-40 and 1% Na_2SO_3) at a ratio of 1:10 (w/v). This stock sap was subsequently diluted to 1:50 and 1:100 in extraction buffer. The wells of microtitre plates were coated with 100 µL of YMV polyclonal antibody diluted 1:1000 in coating buffer (0.05 M sodium carbonate buffer, pH 9.6). The wells were blocked by incubating 200 µL of 5 % Marvel skimmed milk powder (Premier Foods, UK) in each well at 37°C for 1 hour. After emptying the wells (washing not required), 200 µL of each sap dilution was loaded in duplicate wells. Following sap incubation at 4°C overnight and washing, 100 µL of YMV monoclonal antibodies diluted in PBS-T was incubated in each wells followed by 100 µL of goat anti-mouse alkaline phosphatase (Sigma-Aldrich, UK) diluted at 1:40,000 in conjugate buffer (half strength PBS containing 0.05% v/v Tween-20, 0.02% w/v egg albumin, 0.005 mM PVP-40). The alkaline phosphatase conjugate was detected by adding 200 µL p-nitrophenyl phosphate substrate diluted 1 mg ml^{-1} in substrate buffer (97 mL Diethanolamine in 800 mL distilled water pH 9.8) to each of the wells.

The PAS-ELISA followed the method described by Edwards and Cooper [12]. Wells of microtitre plates were coated with 100 µL of protein-A (Sigma-Aldrich, UK) diluted at 1 µg ml^{-1} in coating buffer. Each dilution of the test saps, obtained as described in TAS-ELISA, was sandwiched between YMV rabbit polyclonal antibody diluted 1:1000 in PBS-T. Protein-A alkaline phosphatase (100 µL) diluted at 1:50,000 in conjugate buffer was subsequently added to each well and detected with p-nitrophenyl phosphate substrate as described in the TAS-ELISA.

Both in TAS-ELISA and PAS-ELISA, the microtitre plates were incubated at 37°C for 2 hours after addition of each reagent and washed three times at three minutes intervals using phosphate buffered saline (137.00 mM NaCl, 1.46 mM KH2PO4, 7.75 mM Na2HPO4, 2.68 mM KCl, pH 7.4) containing 0.05 % (v/v) Tween-20 (PBS-T). The only exception was during the blocking step in TAS-ELISA where the plates were incubated at 37°C for 1 hour and the plates were not washed prior to addition of test/control sap. The absorbance (A_{405}) of each well was measured in a DYNEX MRX microplate reader after one hour incubation.

ImmunocaptureReverseTranscriptionPolymeraseChain Reaction (IC-RT-PCR): For IC-RT-PCR, antigen trapping in PCR tubes was achieved as described in TAS-ELISA above and RT-PCR was done as described by Mumford and Seal [7] using the YMV specific primers forward 5'-ATC CGG GAT GTG GAC AAT GA-3' and reverse 5'-TGG TCC TCC GCC ACA TCA AA-3'. Each reaction contained 2.5 U AMV reverse transcriptase (Pomega, USA), 1.25 U GoTaq DNA polymerase (Promega, USA), 0.25 mM of each dNTP (Promega, USA) and 25 pmols of each primer. The cocktail (25 µL) was dispensed into each pre-coated tube, placed in a PTC-200 Peltier Cycler (MJ Research Inc., USA) and subjected to the following thermocyclic regime; 50°C for 10 min for reverse transcription, 95°C for 4.5 min, 95°C for 30 s, 55°C for 1 min and 72°C for 1 min. The cycle was repeated 34 times from the third step followed by a final extension step of 72°C for 10 min. IC-RT-PCR products (10 µL) were analysed on a 1.5 % agarose gel in TAE buffer (40 mM Tris-acetate pH 8.3, 1 mM EDTA) at 100 V for 1 hr. For size estimation, the 1-Kb plus DNA ladder (Invitrogen, USA) was ran along with the PCR products. The gel was stained in ethidium bromide and visualized on a UV-transilluminator.

Table 1: *Yam mosaic virus* (YMV) detection in yam tissues by triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA), Protein A-sandwich (PAS) ELISA and immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) at 1/10, 1/50 and 1/100 sap dilutions.

1/10			1/50			1/100		
TAS	PAS	IC-RT-PCR	TAS	PAS	IC-RT-PCR	TAS	PAS	IC-RT-PCR
+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+
+	+	+	-	+	+	-	+	+
+	+	+	-	-	+	-	-	+
+	-	+	-	-	+	-	-	+
+	-	+	-	+	+	-	+	+
-	+	+	-	+	+	-	+	+
-	-	+	-	-	+	-	-	+
-	-	+	-	-	+	-	-	+
-	-	+	-	-	+	-	-	+
-	-	+	-	-	+	-	-	+
-	+	-	-	+	-	-	+	-
-	+	-	-	+	-	-	+	-
-	+	-	-	+	-	-	+	-
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-	-	-	-	+	-	-	-	-

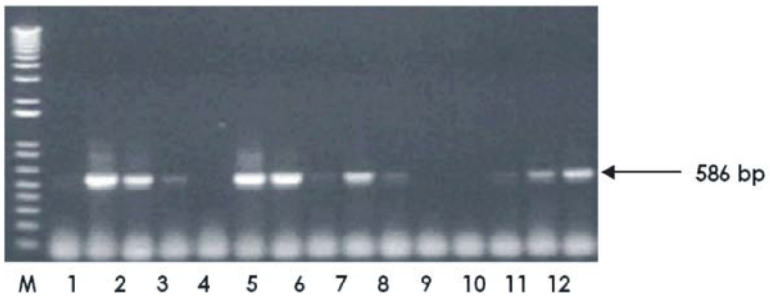

Fig. 1: Gel electrophoresis showing 586 bp of immunocapture-reverse transcription-polymerase chain reaction amplification products for *Yam mosaic virus* (YMV) detection. Lanes 2, 3, 4, 6, 7, 8, 9, 10, 13, 14 and 15 represent YMV positive leaf samples, lanes 1, 5, 11 and 12 represent YMV negative samples. M is 1 Kb plus DNA marker (Invitrogen).

RESULTS

A test is considered positive by ELISA if the mean absorbance (A_{405}) value is twice or more than the mean value of the negative control. While for IC-RT-PCR, any sample that produced the expected 586 bp amplicon is regarded as positive. Of the three detection methods evaluated, IC-RT-PCR was the most sensitive followed by PAS-ELISA. TAS-ELISA was the list sensitive. All eighteen previously tested YMV positive samples re-evaluated in this study reacted positive to YMV by TAS-ELISA, PAS-ELISA and/or by IC-RT-PCR in at least one of the three sap dilutions tested (Table 1, Figure 1). Both at 1/10 and 1/50 dilutions, PAS-ELISA and IC-RT-PCR detected YMV in 11 (61.1%) and 12 (66.7%) of the leaves respectively. Virus detection by PAS-ELISA reduced to 50% at 1/100 dilution while virus detection by IC-RT-PCR increased to 77.8% at 1/100 sap dilution (Figure 2). In TAS-ELISA, YMV was detected in 7 (38.9%) of the leaf samples at 1/10 sap dilution and in 3 (16.7%) samples at 1/50 and 1/100 dilutions (Figure 2). Five of the leaf samples were positive in all three assays at 1/10 dilution, three of these five also tested positive to YMV in all three assays and at all three dilutions (Table 1). Four samples produced the expected 586 bp amplicon by IC-RT-PCR at all sap dilutions but were not detected by any of the ELISA tests. Four other samples reacted positively to YMV antibody by PAS-ELISA at various sap dilutions but reacted negatively by TAS-ELISA and by IC-RT-PCR. All other test samples were positive to at least two of the assays at various sap dilutions (Table 1).

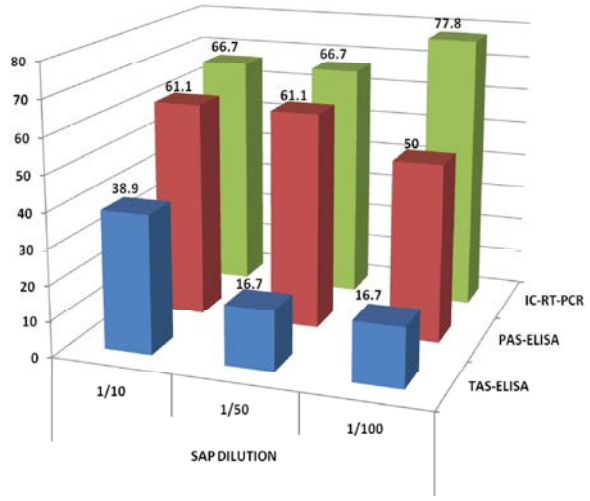

Fig. 2: Detection sensitivity of *yam mosaic virus* (YMV) in yam leaf tissues by triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA), Protein A-sandwich (PAS) ELISA and immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) at 1/10, 1/50 and 1/100 sap dilutions.

DISCUSSION

The superior sensitivity observed in IC-RT-PCR detection of YMV compared to TAS-ELISA and PAS-ELISA is in agreement with previous studies [7, 9, 14] and reaffirms our knowledge of the superior detection sensitivity of the nucleic acid-based techniques over the protein-based detection techniques for plant virus detection [14, 15]. Similar superior detection sensitivity was reported for IC-PCR detection of *Banana streak virus* (BSV), genus *Badnavirus* on *Musa* leaves [16]. The failure of IC-RT-PCR to detect YMV in four of these previously tested YMV positive leaf tissues at all sap dilutions may be attributed to viral RNA deterioration resulting from prolonged storage of the leaf tissues. The yam leaves used in this study were collected during field surveys in Benin and Ghana in 2005. The samples were processed and stored the same day they were collected by cutting them into tiny bits with sterile blades and rapidly drying in tight tubes containing anhydrous calcium chloride and non-absorbent cotton wool. Another possible explanation for the negative results obtained by IC-RT-PCR for these known YMV positive leaf tissues may be due to high viral concentration in the test sap since the samples are dried and the concentrations in the dry weight of the leaf may be more than that in a normal lea weight. This may also account for IC-RT-PCR detection of YMV in two leaf samples at 1/100 dilution but not at 1/10 and 1/50 dilutions (Table 1). The negative results obtained by IC-RT-PCR may also be due to interference of polyphenols and glutinous polysaccharides contained in yam leaves [17].

PAS-ELISA showed greater detection sensitivity compared to TAS-ELISA for the detection of YMV in all three sap dilutions. PAS-ELISA detected YMV in 3 times as many leaf samples as TAS-ELISA at 1/50 and 1/100 sap dilutions (Figure 2). The greater sensitivity observed in PAS-ELISA may be attributed to the use of protein-A in PAS-ELISA. The initial protein-A coating of the microtitre wells before addition of the YMV trapping polyclonal antibody ensures that the antibodies are specifically oriented for antigen binding by binding of the protein-A to the Fc region of the antibody so that the Fab potions, which are required for antigen interaction, are available for antigen binding [12]. An increase in the proportion of the antibodies that are appropriately aligned for virus trapping invariable increases the sensitivity of the PAS-ELISA over the TAS-ELISA where the trapping antibody orientation are not purposely enhanced. The use of protein-A for proper orientation and improvement in the functionality of antibodies is well documented [18-20]. The lower sensitivity of TAS-ELISA may also be due to the use of a monoclonal detecting antibody which may be YMV strain specific. Although group-specific and virus-specific monoclonal antibodies have been reported for members of the potyvirus group, strain specific monoclonal antibodies have also been reported [21, 22]. Thus, based on the results of this studies, we conclude that the current YMV monoclonal antibody is YMV strain specific, an attribute which maybe useful for serotyping and differentiation of YMV strains. Our results however clearly confirm that TAS-ELISA is not suitable as a sole diagnostic method for YMV. This finding may necessitate re-evaluation and re-grouping of previously characterized yam varieties and accessions where TAS-ELISA was the sole diagnostic technique used in deciding the resistant, susceptible or tolerant status of such yam varieties and accessions.

We recommend that where resources are available IC-RT-PCR should be used for YMV diagnosis and certification. However, given that ELISA is easier, less expensive and less time consuming, the use of PAS-ELISA at 1/10 (w/v) sap dilution is recommended for routine YMV screening where resources are limited.

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