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Full Length Research Paper

Diversity of banana streak-inducing viruses in Nigeria and Ghana: Twice as many sources detected by immunoelectron microscopy (IEM) than by TAS-ELISA or IC-PCR

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Our previous study had shown that some *Musa* leaf samples with *Banana streak* symptoms tested negative *for Banana streak virus* (BSV) in triple antibody-sandwich ELISA (TAS-ELISA). Therefore, in this study 63 additional *Musa* leaf samples were tested for BSV by TAS-ELISA, immunoelectron microscopy (IEM) and immunocapture polymerase chain reaction (IC-PCR). Sensitivity tests by sap dilution end-point analyses indicated that IC-PCR was considerably more sensitive than IEM for detecting typical BSV, while IEM proved to be of similar sensitivity as TAS-ELISA. However, when leaf samples of *Musa* plants, obtained from different farmers' fields in Nigeria and Ghana and some Nigerian sources maintained in the greenhouse were screened for BSV, more than twice as many samples revealed BSV-like particles by IEM than were detected by TAS-ELISA or IC-PCR. Of the 51 leaf samples that were BSV positive in all tests taken together, 48 were positive by IEM, 25 by IC-PCR and only 19 by TAS-ELISA. Upon IEM examination, typical bacilliform BSV-like particles were clearly recognized although in very diverse concentrations. Bacilliform particles deviating in length from the main particle populations or showing an angularly bent morphology were found. Occasionally, in certain samples and with certain antisera the IEM decoration tests revealed mixtures of strongly decorated and weakly decorated BSV-like particles or bacilliform particles which did not at all react with the antibodies available. This proved, the occurrence, besides the presence of typical BSV, of diverse populations of BSV-like viruses in West Africa

Key words: *Banana streak virus*, diversity, serological assays, immunoelectron microscopy, immunocapture PCR.

INTRODUCTION

Banana streak virus (BSV), a plant virus with bacilliform particles and a circular dsDNA genome of 7.4 kbp, is a member of the genus *Badnavirus* in the family *Caulimoviridae*. It is causal agent of banana streak disease which occurs worldwide in banana and plantains (*Musa* spp.) and which is widespread in Africa. Estimated yield losses of between 7-90% have been attributed to the disease (Harper et al., 2004; Lockhart et al., 1998; Davis et al., 2000; Daniells et al., 2001). Most recently, genetic variants of BSV have been reported in Mauritius (Jaufeerally-Fakim et al., 2006).

Banana streak virus (BSV) integrated to the Musa) host genome (Lockhart et al., 1998; Ndowora et al., 1999; Harper et al., 1999; Geering et al., 2001 making nucleic-

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acid-based detection difficult to interpret. However, with immnocapture polymerase chain reaction (IC-PCR) developed by (Harper al., 1998), it is now possible to specifically detect and differentiate the encapisdated, episomal (particulate) form from the integrated counterpart.

A large diversity exists among the virus sources inducing BSV-symptoms which are currently subsumed under the species BSV. Five serologically and genomically distinct naturally occurring isolates of BSV have been identified from Costa Rica, Honduras, Morocco, Rwanda and Trinidad (Lockhart, 1994; Ndowora and Lockhart, 2000; Harper et al., 2004). An extensive genetic variation among isolates of BSV from Australia was determined by sequence comparison of the conserved sequence motifs of the badnavirus replicase (Geering et al., 2000). Diversities among several isolates reached an extent comparable to genetic distances separating other badnavirus species. Recent research of Harper et al. (2004) described the complex situation of the banana streak disease in Uganda, an important banana growing region of Africa, where also a large number of variants of BSV have been identified. Even, more recently, using Badna degenerate primers for the conserved reverse transcriptase/RNase H region of the badnavirus open reading frame (ORF) III, 36 distinct sequences out of 103 clones of amplified *Musa* cultivars showed less than 85% nucleotide identity to each other, undermining the variability of BSV (Geering, 2005).

The large genomic diversity of BSV presents a significant problem to virus indexing and to the prescription of a general diagnostic protocol. Already Lockhart and Olszewski (1993) recognized the existence of different serotypes of BSV and the limitation of the use of serological assays in virus indexing. To broaden the spectrum of BSV variants detected by serological assays, a cocktail of antibodies (PMX2RC) against different isolates of BSV was produced (Ndowora and Lockhart, 2000). With many different new BSV isolates been detected, there was the need for less-serotype dependent serological diagnostic approach to BSV indexing.

But recent studies of Agindotan et al. (2003) showed that BSV monoclonal and polyclonal antibodies raised against a Nigerian isolate of BSV did not detect the virus by TAS-ELISA in several *Musa* leaf samples with characteristic yellow streak symptoms of BSV, so there could be more than one strain in Nigeria. In this paper we show variation patterns of BSV isolates from Nigeria and Ghana.

MATERIALS AND METHODS

Monoclonal antibodies and antisera

Monoclonal antibodies (Mabs) to BSV, BSV-IITA 3F9/1 and BSV-IITA 3D4/2, the mouse antiserum BSV-IITA and the rabbit antiserum BSV-IITA were raised against a Nigerian BSV isolate purified from a plantain hybrid, TMPx 7002-1 (Agindotan et al., 2003). The antibody cocktail from rabbit antisera to BSV (PMX2RC) was kindly supplied by Prof. B. Lockhart (Ndowora and Lockhart , 2000), were used for BSV indexing. The DSMZ TAS-ELISA kit T-0475-491/1 (DSMZ, Brauschweigh, Germany) was used for detection of *Cucumber mosaic virus* (CMV).

Determination of sap dilution end-points in IEM, IC-PCR and TAS-ELISA

The sensitivity of IEM, IC-PCR and TAS-ELISA for the detection of BSV was evaluated by determination of dilution end-points of plant extract from a BSV-infected tropical *Musa* plantain hybrid (Sample # 20 in Table 1). BSV-infected or healthy plantain leaf tissue was ground 1:4 (w:v) in BSV extraction buffer; [phosphate buffered saline (100 mM phosphate buffer, pH 7.4, + 15 mM NaCl), (PBS) (Clark and Adams, 1977), containing 2% polyvinyl pyrrolidone (PVP 15 000) and 1% Na_2SO_3]. Leaf extracts were further diluted in PBS in a three-fold series and subjected to IEM, TAS-ELISA and IC-PCR for detection of BSV.

Triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA)

The sensitivity of TAS-ELISA for detecting BSV was determined using the TAS-ELISA procedure described by Thottappilly et al. (1998). Rabbit BSV-IITA IgG at 2 µg/ml was used for coating microtitre plates. Serially diluted sap extracts of healthy and infected leaves were added. A mixture of the MAbs in culture medium, BSV 3F 9/1 diluted 1:250 in PBS, and BSV 3D4/2 undiluted, was used as secondary antibodies. In another screening, BSV-IITA mouse polyclonal antibodies diluted 1:30000 in PBS was also used as secondary antibodies. A rabbit-anti-mouse alkaline phosphatase conjugate (DAKO A/S, Denmark) diluted 1:1000 in conjugate buffer was used as reporter and 1 mg/ml p-nitrophenyl phosphate [Sigma 104-105] as substrate, were used. ELISA mean absorbance readings at 405 nm of the tested samples greater than twice the mean of healthy negative control, were considered positive for BSV, while those with values below were considered negative for the virus.

Immunoelectron microscopy (IEM)

The IEM detection of particles always comprised a trapping step which was in certain cases combined with an identification step (decoration test). Nickel grids (400-mesh) coated with Formvar/carbon films were floated on ca. 15 µl drops of BSV antiserum (mouse or rabbit) diluted 1:1000 in 0.1 M phosphate buffer pH 7.0 (PB) for 5 min at 20°C. After washing with 20 drops of PB, antibody-coated grids were floated on ca. 15 µl drops of ca. 1:20 diluted leaf sap extracts in BSV extraction buffer and incubated overnight at 20°C. After rinsing with 40 drops of distilled water, each grid was negatively stained with three drops of 1% aqueous uranyl acetate, drained with filter paper and dried. Virus particles were observed in a transmission electron microscope (ZEISS 906E, Carl Zeiss, Oberkochem, Germany).

For IEM plus decoration, the particle trapping was performed as described above but after the incubation with leaf extracts the grids were washed with 20 drops of PB and each grid was floated for 15 min at 20°C on ca. 15 µl drops of 1:50 diluted antiserum cocktail or rabbit or mouse BSV-IITA antiserum or culture supernatant of MAbs.

Immunocapture polymerase chain reaction (IC-PCR)

Immunocapture-PCR was done essentially as described by Harper et al. (1998), using BSV-Onne specific primers. Microcentrifuge

Table 1. Detection of Banana streak virus (BSV) in field and greenhouse samples of banana and plantains using Triple antibody-sandwich enzyme-linked immunosorbent assay (TAS-ELISA),double-antibody enzyme-linked immunosorbent assay (DAS-ELISA), immunosorbent electron microscopy (IEM) and immunocapture polymerase chain reaction (IC-PCR).Different reaction types are separated by single lines. Hatched lines indicate samples selected for tests in Table 2.

Musa sample	Symptoms	Location	ELISA[a] TAS-BSV MAb 3F9/1+ 3D4/2	ELISA[a] TAS-BSV Mouse IITA	IEM[b] BSV Rabbit IITA	IC-PCR BSV Rabbit IITA	IEM[b] Tubular struct.	IEM[b] Bullet shaped struct.	ELISA[a] CMV
1	Yellow streak	DSMZ, Onne, Nigeria	+++	+++	+++++	+	+	-	-
4	Yellow streak	DSMZ, Onne, Nigeria	+++	+++	++++	+	+	-	-
6	Yellow streak	DSMZ, Onne, Nigeria	+++	+++	++++	+	++	-	-
7	Yellow streak	DSMZ, Onne, Nigeria	+++	+++	++++	+	++	-	-
8	Asymptomatic	DSMZ, Onne, Nigeria	+++	+++	++++	+	++	+	-
9	Asymptomatic	DSMZ, Onne, Nigeria	+++	+++	+++	+	++	-	-
10	Asymptomatic	DSMZ, Onne, Nigeria	+++	+++	++++	+	++	++	-
11	Asymptomatic	DSMZ, Onn, Nigeria	+++	+++	+++	+	+	++	-
13	Yellow streak	DSMZ, Onne, Nigeria	+++	+++	+++	+	+	-	-
17	Yellow streak	IITA, Nigeria	+++	+++	+++	+	++	++	-
18	Yellow streak	IITA, Nigeria	+++	+++	+++	+	-	++	-
19	Yellow streak	IITA, Nigeria	+++	+++	++	+	+++	-	-
20	Yellow streak	IITA, Nigeria	+++	+++	+++	+	++	++	-
24	Yellow streak	IITA, Nigeria	+++	+++	+++	+	++	-	-
25	Yellow streak	Ibadan, Nigeria	+++	+++	+++	+	+++	-	-
41	Yellow streak	Ondo, Nigeria	+++	+++	+	+	+	-	-
47	Yellow streak	Ado-Ekiti, Nigeria	+++	+++	+	+	+	-	-
37	Yellow streak	Ijebu-Ode, Nigeria	-	-	+++	+	++	++	-
44	Yellow Streak	Ondo, Nigeria	-	-	+++	+	+	+	-
32	Yellow streak	Ibadan, Nigeria	-	-	+++	+	++	-	-
42	Yellow streak	Ondo, Nigeria	-	-	+++	+	+	+	-
26	Yellow streak	Ibadan, Nigeria	-	-	++++	-	++	++	-
30	Yellow streak	Ibadan, Nigeria	-	-	+++	-	+++	-	-
33	Yellow streak	Ibadan, Nigeria	-	-	++	-	++++	++	-
34	Yellow streak	Ibadan, Nigeria	-	-	++++	-	+++	-	-
35	Yellow streak	Ibadan, Nigeria	-	-	++	-	++	++	-
39	Yellow streak	Ijebu-Ode, Nigeria	-	-	+++	-	++	-	-
40	Yellow streak	Ondo, Nigeria	-	-	+++	-	++	-	-
27	Yellow streak	Ibadan, Nigeria	-	-	+++	-	++	++	-
36	Yellow streak	Abeokuta, Nigeria	-	-	+++	-	+++	+++	-
38	Yellow streak	Ijebu-Ode, Nigeria	-	-	+++	-	++	-	-
59	Yellow streak	Ghana	-	-	++++	-	-	+	+++
2	Yellow streak	DSMZ, Onne, Nigeria	-	-	++++	-	-	-	+++
12	Asymptomatic	DSMZ, Onne, Nigeria	-	-	++++	-	+	-	-
61	Asymptomatic	Ghana	-	-	+++	-	+	-	-
22	Yellow streak	IITA, Nigeria	-	-	++	-	+	+	-
48	Yellow streak	Ghana	-	-	+	-	+	-	-
46	Asymptomatic	Ado-Ekiti, Nigeria	-	-	+	-	+	-	-
28	Yellow streak	Ibadan, Nigeria	-	-	+	-	++	-	-
29	Yellow streak	Ibadan, Nigeria	-	-	+	-	++	-	-

Table 1. Continued.

5	Asymptomatic	DSMZ, Onne, Nigeria	-	-	+	-	++	-	-
52	Yellow streak	Ghana	-	-	+	-	-	-	+++
53	Yellow streak	Ghana	-	-	+	-	-	+	+++
58	Yellow streak	Ghana	-	-	+	-	+	-	+++
51	Yellow streak	Ghana	-	-	+	-	+	-	+++
55	Yellow streak	Ghana	-	-	+	-	+	-	+++
57	Yellow streak	Ghana	-	-	+	-	+	-	+++
45	Asymptomatic	Ado-Ekiti, Nigeria	-	-	-	+	-	-	-
43	Asymptomatic	Ondo, Nigeria	-	-	-	+	-	-	-
31	Asymptomatic	Ibadan, Nigeria	-	-	-	+	+++	-	-
49	Yellow streak	Ghana	-	-	-	-	+	+	+++
50	Yellow streak	Ghana	-	-	-	-	+	+	+++
54	Yellow streak	Ghana	-	-	-	-	+	-	+++
56	Yellow streak	Ghana	-	-	-	-	+	+	+++
60	Yellow streak	Ghana	-	-	-	-	+	+	+++
15	Yellow streak	IITA, Nigeria	-	-	-	-	++	++	+++
3	Asymptomatic	DSMZ, Onne, Nigeria	-	-	-	-	-	-	-
14	Bunchy top	DSMZ, Australia	-	-	Nt	-	Nt	Nt	-
16	Bunchy top	DSMZ, Australia	-	-	Nt	-	Nt	Nt	-
21	Bunchy top	DSMZ, Australia	-	-	Nt	-	Nt	Nt	-
23	Asymptomatic	IITA, Nigeria	-	-	-	-	-	-	-
62	Asymptomatic	Ghana	-	-	-	-	+	-	-
63	Ensete, negative control	DSMZ, Ethiopia,	- 0.058	- 0.034	-	-	-	-	- 0.041

[a] ELISA tests: scoring of mean absorption values (A_{405}nm): - = <x2 mean healthy control; + = >x2 mean but <x3 healthy control ; +++ = >x4 mean healthy control

[b] Immunosorbent electron microscopy (IEM): particle numbers per screen at 40 000 X magnification: - = no, + = 0.01-0.1, ++ = 0.1-1, +++ = 1-10, ++++ = 10-100, +++++ = 100-1000 virus particles

[c] Samples of hatched fields have been further tested in Table 2.

Nt = Not tested.

tubes (0.2 ml) were each coated with 100 µl of 2 µg/ml BSV-IITA rabbit IgG in coating buffer and incubated at 37°C for 2 h. Another set of tubes was coated with 100 µl per tube of 2 µg/ml BSV PMX 2RC. Test tubes were washed three times with PBS-T (PBS + 0.05% Tween 20) followed by addition of 100 µl each of serially diluted leaf sap extracts and incubated overnight at 4°C. After removing the sap extracts and washing the tubes with PBS-T, 50 µl of PCR reaction mix was added. This consisted of 3 µl each of 10 µM BSV primers (BSV 4673: 5' GGA ATG AAA GAG CAG GCC 3', BSV 5317: 5' AGT CAT TGG GTC AAC CTC TGT CCC 3' (BSV-Onne primers; Harper et al., 1998), 1 µl of 25 mM dNTPs, 5 µl of x10 *Taq* polymerase buffer, 5 µl of 5 U/µl *Taq* polymerase (INVITROGEN), 2.5 µl 50 mM $MgCl_2$ and 34 µl of sterile double distilled water. The amplification was done at 95°C for 3 min, 35 cycles of 95°C for 1 min, 53°C for 1.5 min and 72°C for 2 min, followed by an extension at 72°C for 10 min. All PCR products were separated by electrophoresis in 1% agarose gels containing 0.5 µg/ml of ethidium bromide in TAE (40 mM Tris-acetate, 0.001 M EDTA, pH 8.0).

Screening of Musa leaf samples for BSV by IC-PCR, IEM and ELISA

Banana and plantain leaves exhibiting yellow streak symptoms and several asymptomatic leaves were collected from road side plants and from home gardens in Ondo, Oyo, Ekiti and Ogun states of Western Nigeria. Samples were also collected in *Musa* trial fields of IITA in Ibadan, Nigeria. Banana leaves collected in Ghana were included in the virus indexing. Several BSV infected plantains from Onne, Nigeria, maintained in the glasshouse at the DSMZ Plant Virus collection in Braunschweig, Germany, tested and other samples infected by banana bunchy top virus and an uninfected plant of Ensete served as negative controls (Table 1).

Leaf samples were ground 1 to 4 (w/v) in BSV extraction buffer and tested by TAS-ELISA and IEM. There was no enrichment step or partial virus purification protocol applied prior to the virus detection assays. TAS-ELISA was performed as described above using the monoclonal antibody mix, and the mouse antiserum diluted 1:3000 (v/v) in PBS as detecting antibody and the rabbit BSV-IITA antiserum for coating. CMV was tested by TAS-ELISA

essentially as recommended by the DSMZ Plant Virus Division, except that 100 µl reaction volumes were used for the virus assays.

Screening of *Musa* samples for BSV by IEM was as described above with the rabbit antiserum BSV-IITA for trapping and decoration and in certain cases purified cocktail rabbit polyclonal antibodies (PMX 2RC) was used in comparison for decoration. The *Musa* samples were tested for BSV by IC-PCR as described above.

RESULTS

Relative sensitivity of three assays in detecting BSV in crude plant extracts

Detection of BSV in infected *Musa* leaf tissue by IC-PCR was still possible at sap dilution of over 1:32,000. BSV was detected at sap dilutions as low as 1:3645 and 1:1215, by IEM and TAS-ELISA, respectively.

Screening of Musa samples from different locations for BSV

Out of the 63 *Musa* plants tested (Table 1), 50 were positive for BSV or BSV-like viruses (eight of these positive to both CMV and BSV), six for CMV only. Eight samples were negative to either BSV/BSV-like viruses or CMV. These eight plants did not show any leaf streak symptoms (three of them were DSMZ control plants infected by *Banana bunchy top virus*, one Ensete, four were asymptomatic field samples). All plants with CMV infections showed streak symptoms.

Of the 50 different samples that tested positive for BSV/BSV-like viruses with all the tests combined, 47 were positive by IEM. High particle numbers between 10 and more than 100 per viewing field of the electron microscope indicated an effective specific serological binding by the coating antiserum from relatively high concentrations in the leaf extracts with 33 samples. On the other hand low amounts of particles in 11 samples might indicate a very ineffective trapping caused either by a low reactivity of these virus sources with the BSV antiserum or extremely low particle concentrations in the crude extracts compared to the 33 samples above. Only in 24 and 19 samples, respectively, BSV infections were detected by IC-PCR and by TAS-ELISA. All TAS-ELISA positives were also positive by IEM and IC-PCR. However, two samples were positive only in IC-PCR (Table 1).

Correlation of detection results with leaf symptoms

Out of the 47 plant samples positive in IEM, 39 had conspicuous yellow streak symptoms (including seven with additional CMV infections) while eight were asymptomatic. Of these asymptomatic plants, six had been grown in the DSMZ glasshouse in Germany but were originally collected as symptomatic samples in Nigeria (Onne region). Only two asymptomatic but IEM positive samples were collected in Nigeria and Ghana (sample 46 and 61, Table 1). Seven of the asymptomatic but IEM-positive samples revealed high particle numbers in IEM. Only 17 of 24 samples, BSV-positive in IC-PCR, showed symptoms, while seven were asymptomatic. Four of these samples were the same from the DSMZ glasshouse, which contained bacilliform particles. But the other three gave PCR reactions only and did not reveal BSV-like particles and no ELISA reaction. The IC-PCR results were the same whether BSV-IITA rabbit poly, BSV-IITA mouse polyclonal or PMX 2RC were used for trapping BSV.

Of the 47 samples which contained bacilliform particles, two contained only BSV particles, whereas 45 showed BSV particles accompanied by tubular or bullet-shaped components of variable appearances and unknown origin (described in more detail below). Both component types and their numbers did not appear to be associated with BSV-like particles. Of the 17 samples without bacilliform BSV-like particles, eight also showed these components. Thus they were supposed to be constituents of banana cells of non-viral origin.

Morphology of BSV-particles and the tubular and bullet-shaped particle-like components

In all samples tested positive by IEM and especially in leaf sample number 10 (Table 1), which was selected for studying morphological details, the bacilliform particles, 28-30 nm in diameter and 130 nm in length, were predominantly found (Figure 1a). Only rarely longer bacilliform particles of up to 2000 nm were also found (Figures 1f and g). Generally, BSV particles were uniformly negatively stained but in some cases the stain was intense longitudinally across the inside of the virus (see electron-dense internal parts in Figures 1b, c and g). Angularly bent BSV particles showing different degrees of inclination between their arms were also frequently observed (Figures 1a - e), although at low incidences.

As mentioned above, most IEM preparations also revealed different types of conspicuous tubular (Figure 2) or bullet-shaped (superficially resembling to rhabdovirus particles) particle-like components (Figure 2f - i). The tubules and the bullet-shaped components had varying lengths and a width of ca. 60 – 80 nm (about twice the width of bacilliform BSV particles) and were mostly ca. 270 - 330 nm long. However, an internal layered fine structure typical of penetrated rhabdovirus particles was not visible in the bullet-shaped components when stained with either uranyl acetate or ammonium molybdate or after fixation with glutaraldehyde (Figure 2i). Since a rhabdovirus-like internal structure was never resolved, it was concluded that the conspicuous tubular and bullet-shaped particles cannot be considered as of viral origin.

As mentioned above, both components have no direct association with the BSV-like infections.

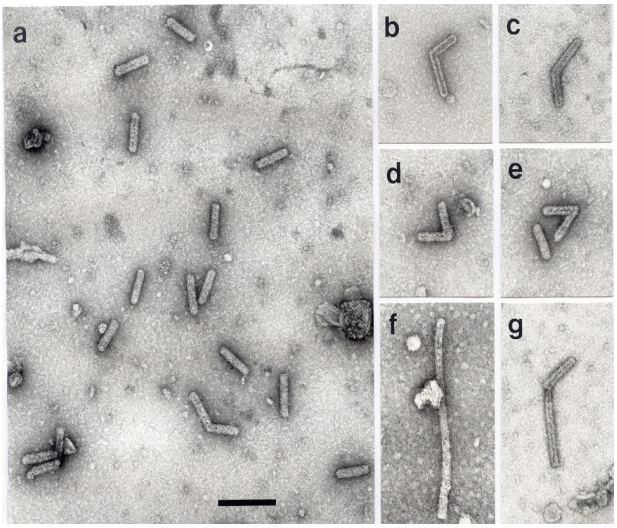

Figure 1. Morphology of different types of typical BSV-like bacilliform particles (a and f) and angularly bent particles with various degrees of inclination (a-e) after trapping on BSV-IITA antiserum coated grids. Examples of elongated particles are shown in f and g. All negatively stained with uranyl acetate. All micrographs at identical magnification, bar equals 200 nm.

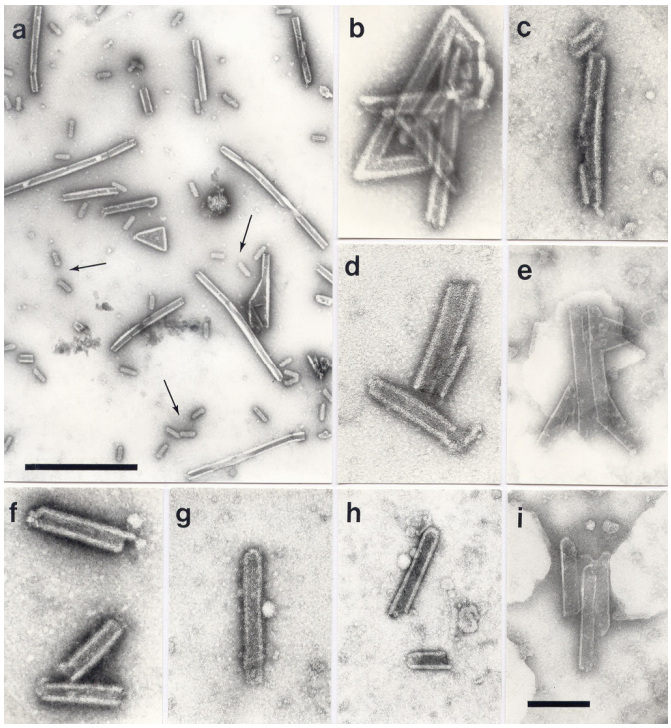

Figure 2. Morphology of non-viral tubular and bullet-shaped particles in crude leaf extracts of Banana streak virus-infected plant sample Nb. 10, trapped on a grid coated with IITA-BSV antiserum (a) bacilliform BSV-like particles (arrows) and tubular structures; (b-e) tubular particles; (f-i) bulletshaped particles. (e) and (i) negatively stained with ammonium molybdate, all others with uranyl acetate. Magnification bars equal 1000 nm (a) and 200 nm (b – i).

Serological analysis of isolates, antisera and monoclonal antibodies

Thirteen samples reacted strongly in ELISA and IEM tests. Only in about one-half of the samples containing BSV-like particles, was a strong immunotrapping of particles observed with both of the polyclonal antisera (Table 1). When the two antisera and two Mabs were compared in IEM + decoration in their activity against six selected infected samples, Lockhart's cocktail of rabbit antibodies (PMX 2RC) and the rabbit antiserum BSV-IITA lead to similar results in respect of the particle numbers trapped per standard area of the grid from five of the samples whereas the sixth sample was very weakly trapped if at all (Table 2). On the other hand only from two of the five samples MAbs trapped BSV particles, while in four samples no particles were trapped (Table 2). Obviously the Mabs had no specificity for the BSV-like particles present in these four samples. Interestingly the same four samples had also been negative in the ELISA tests shown in Table 1 and samples 27 and 40 were negative also in IC-PCR. These IEM results presented in Table 2, thus, revealed further indications for the differentiation between the viruses infecting these samples.

Similarly, the decoration capacity of the BSV antisera illustrated the serological differentiation of the selected samples (Table 2). While Lockhart's antiserum decorated the BSV-like bacilliform particles in five of the samples strongly, the IITA antiserum decorated the particles strongly in only two of the six samples (Table 2, samples 10 and 40). A uniform strong decoration is exemplified in Figure 2a. However, in sample 10 the decoration test with IITA antiserum revealed, additionally to the many strongly decorated particles, a population of very weakly decorated BSV-like particles as shown in Figure 2d. The heterogeneity of sample 10 was also recognized when the two monoclonal antibodies decorated, although weakly, the larger proportion of particles whereas a smaller proportion of the particles remained undecorated in the same preparations (Figure 2b). The mixed particle population was not visualised by Lockhart's antiserum which decorated all BSV particles in sample 10 (Table 2). The reaction spectrum of sample 26 differed from that of sample 10 because only a uniform population of particles was seen with all four antibodies, and since only Lockharts antiserum cocktail reacted strongly whereas the IITA antiserum reacted very weakly, like the two Mabs (Figure 2b and c). Additional differentiated decoration patterns were recognized with the other four samples in Table 2. Among these, the decoration with samples 27 and 44 appeared identical, but samples 40 and 37 were each differing from the former and from each other. Sample 37 contained BSV-like particles which could not be decorated by any of the antibodies involved in this study. The various differential decorations pattern of the

Table 2. Immunoelectron microscopical differentiation of selected banana leaf samples with streak symptoms: trapping and decoration intensities of the bacilliform BSV-like particles using antisera and monoclonal antibodies.

Musa sample number	Results of diagnostic tests in Table 1	Antibodies/antisera, antibody coating dilution and dilutions for decoration			
		Rabbit antiserum PMX 2RC, IgG trapping 10 µg/ml decoration 1 mg/ml	Rabbit antiserum BSV-IITA trapping 1:1000 decoration 1:50	MAb BSV-IITA 3D4/2 IgG trapping 10 µg/ml decoration 1 mg/ml	MAb BSV-IITA 3F9/1 IgG trapping 10 µg/ml decoration 1 mg/ml
10	IEM + ELISA + IC-PCR +	+++++ strong [b]	+++++ strong [b] ++ weak [c]	+++ weak [b] ++ no [c]	++ weak [b] ++ no [c]
26	IEM + ELISA - IC-PCR -	++++ strong	++++ weak	++++ weak	++++ weak
27	IEM + ELISA - IC-PCR -	+++ strong	+++ weak	0	0
44	IEM + ELISA - IC-PCR +	+++ strong	+++ weak	0	0
40	IEM + ELISA - IC-PCR -	++ strong	++ strong	0	0
37	IEM + ELISA - IC-PCR +	+ no	+ no	0	0
3, control	IEM - ELISA - IC-PCR -	0 [a]	0	0	0

[a] Numbers of particles trapped on antiserum/antibody coated grids per electron microscope screen scored at 40,000 X magnification: 0 = no particles found; + = 0.01-0.1; ++ = 0.1-1; +++ = 1-10; ++++ = 10-100; +++++ = 100-1000 particles
[b] Scoring of decoration intensities in words: no, weak or strong
[c] Scoring of decoration intensities in words: no. Weak or strong, for a second population of the virus in the sample

particles in the six selected banana samples suggest the existence of at least five different antigen types.

DISCUSSION

Morphology of BSV and BSV-like particles

For the main particle population, we measured a size of 29 x 130 nm, which coincides very well with ca. 30 x 130-150 nm as recently reported for BSV by Geering and Thomas (2002). Exceptionally long particles, up to 2000 nm, were observed in our preparations like has also been reported before for BSV (Geering and Thomas, 2002) and other badnaviruses (Hull et al., 2004). Brunt et al. (1964) interpreted the long particles of CSSV as multiples of the modal length. In additional to the long particles, we relatively often observed angularly bent 'bacilliform' particles. Similar angularly bent particles, together with abnormally long bacilliform particles (up to 2680 nm), have also been recorded from *Cacao swollen shoot virus* (CSSV), genus *Badnavirus* (Adamako et al., 1983; Brunt et al., 1964). Frequently observed and very conspicuous tubular, sometimes bacilliform or bullet-shaped structures in the crude banana leaf extracts had to be interpreted as non-viral host plant constituents. These structures have shortly already been mentioned by Lockhart (1986).

Correlation between infections and symptoms

We could prove that because of their specific morphology the detection of the bacilliform particles in the IEM is unequivocal and possible at extremely low particle concentration and that we could detect BSV satisfactorily well in crude leaf extracts. From Table 1, it is evident that

in the Nigerian samples, yellow leaf streak symptoms correlated very well with infections by bacilliform particles. Particles could, however, also be detected in nine plants without leaf streak symptoms. Such a correlation could not be checked for samples from Ghana because most of these were infected by cucumber mosaic virus (CMV) that also causes yellow leaf streak symptoms. Nevertheless, more than the half of the Ghanaian samples was infected with both CMV and bacilliform particles. Within the asymptomatic samples from Nigeria as well as from Ghana different antigenic/genomic virus types could be distinguished (Table 1). Some of the leaf streak-expressing plants collected in Nigeria became asymptomatic after cultivation in a glasshouse in Germany but they still contained high virus concentrations. Already Dahal et al. (1998) reported in detail about the variability of symptom expression and BSV detection under different environmental and experimental conditions in Nigeria. The development of leaf streak is not only dependent on infection by certain virus strains/types but also on the culture conditions. It still remains unknown which, and how many, genomic variants are regulating the symptom expression under certain environmental conditions and whether observed asymptomatic infected plants may be indicative of the presence of special genomic virus variants.

Diverse reaction spectra in the samples

In our materials, it appears difficult to determine the exact number of virus variants without analysing the genomic sequences. Nevertheless, we can compare the reaction spectra of our samples on the basis of the four technical approaches; TAS-ELISA applying two different antisera systems, IEM and decoration tests with the rabbit IITA antiserum (and other Mabs and antiserum) and IC-PCR using one of the IITA antisera for immunocapture and the PCR primers designed for BSV Onne (Nigeria) strain (Harper et al., 1998). The results presented in Table 1 reveal much differentiated reaction patterns. Among the 47 samples revealing bacilliform viruses in IEM, three different serological reaction patterns were observed. The first group of 17 samples clearly tested as BSV in TAS-ELISA and in IC-PCR. The second group of 19 samples revealed high particle trapping in IEM but only two of these samples reacted, very weakly, in ELISA, and only four were positive in IC-PCR. Thus, samples of this second reaction type contained high numbers of particles but, nevertheless, tested negative or weakly positive, when using the same antiserum with the two supposed 'routine techniques' ELISA and IC-PCR. A third group of 11 samples contained particles at very low concentrations which appeared to be ineffectively trapped in IEM. Altogether, 30 of the 47 samples contained particles which, apart from very few exceptions, could not be detected by ELISA and/or IC-PCR. Only in three of the samples of Table 1 that IC-PCR signal was obtained but no detection with any other method.

Apparently, the high sensitivity could not be utilized when PCR was applied to highly variable virus populations found with BSV-like viruses, neither within one region nor in different geographical regions. The BSV-Onne primers used were definitely not amplifying all BSV isolates present in the samples tested. This indicated that there were variants of BSV Onne in Nigeria. A further serological differentiation was revealed by the decoration experiments with six of the samples and with four different antibody sources presented in Table 2. The samples belonged to two of the reaction patterns distinguishable on Table 1. If we, additionally, consider all differential reactions in terms of decoration scorings, we have to postulate at least four different reaction patterns within the samples on Table 2. Our results on both Tables, therefore, indicate that in sum at least 7 different virus variants could be distinguished using all the technical options applied in this study. A further complication for the study of the differentiation of leaf streak inducing banana badnaviruses was exemplified by sample 10, for which a mixed infection of two serologically different virus variants is documented (Table 2). Similarly Harper et al. (2004) reported the occurrence of more than one species in certain single plants, and also the detection of some BSV isolates by IEM and not by ELISA, in agreement with our findings.

Virus variants represent several virus species

Until recently the badnavirus variants from banana had been subsumed under the term BSV because of the symptoms induced in the infected plants and their association with bacilliform particles. The exact taxonomical significance of the 'virus variants' recognized in our analyses remains open but our results corroborate former observations on the variability of banana infecting-badnaviruses and the currently developing taxonomical conclusions derived from various genome analyses (for references see below). Thus, we could demonstrate that only 17 of 48 bacilliform particle-positive samples can be interpreted as members of the species BSV including their differentiation in three reaction types. It cannot be decided whether the latter are genomic variants. The other 31 samples containing bacilliform particles deviate so much from ELISA and PCR reactivity as would be expected for members of the virus species BSV that it has to be doubted whether they belong to this virus species. Thus we have to realize that more than half of our samples (including all samples from Ghana) may not belong to the species of BSV.

Previous reports about the variability of bacilliform viruses infecting banana referred to the geographical areas of Nigeria (Lockhart and Olszewski, 1993; Dahal

et al., 1999), Middle America (Costa Rica, Honduras, Trinidad) (Lockhart 1994), East Africa (Harper et al., 1996, 2004) and Australia (Geering et al., 2000). The degree of variation on nucleic acid level and serology had been interpreted already as sufficiently large in order to define certain of the variants as distinct virus species (Geering et al., 2000; Geering and Thomas 2002; Harper et al., 2004; Hull et al., 2004). Geering et al. (2000) concluded that the four Australian isolates analysed might each have the status of a distinct species. Harper et al. (2004, 2005) deduced BSV species in Uganda based on the sequences of amplified PCR products using badna degenerate primers have risen between 12 -15. The variation was even found in the most conserved reverse transcriptase/RNase H region of the badnavirus open reading frame (ORF) III that represents ~10% of the virus genome. More diversity in BSV has been reported (Geering et al., 2005). In the most recent taxonomical report of ICTV, three different species of BSV-like viruses have been approved (Hull et al., 2004). Our observations underline that future intensive sequence studies on a broad collection of samples/isolates from all known banana cultivation regions will be needed for a comprehensive fitting of all badnavirus infections of banana under a common taxonomical system.

Also, in other badnaviruses, high strain/species diversity has been recorded. *Sugarcane bacilliform virus* (SBV) isolates showed high genomic variability (Braithwaite et al., 1996; Hardy et al., 1996). SBV was on the other hand found to be relatively closely related to BSV in terms of serology, host range and genomic properties (Lockhart and Autrey, 1988; Bouhida et al., 1993). Also for cacao swollen shoot virus, a high serological and genetic variability of the different sources has been reported (Muller and Sackey, 2004).

As a consequence of the described diversification among the badnavirus variants, the limitations for indexing the many virus variants are still large although the tools used by us and others are those developed in several previous research attempts for optimization of the detection techniques. These comprise firstly the production of broad spectrum antiserum cocktails. The most powerful, produced by Lockhart, against many BSV isolates, was used in the present study. Recently, specific primers to four Australian badnavirus isolates have been designed. Their application to a broad spectrum of further virus sources are being tested; thus it is not known whether these allow for universal detection of the badnaviruses in banana (Geering et al., 2000). As a consequence for practical applications, we have to realize from our comparative test results as well as from previously published data that current tools neither of serology nor of sequence specific IC-PCR are sufficient in their specificity to serve as the desired diagnostic routine tests for a universal, comprehensive and reliable indexing of banana plants for badnaviruses.

For now, under our conditions only IEM was by far the most sensitive and reliable diagnostic method compared to ELISA or PCR in order to detect bacilliform BSV-like banana infections. However, the detection by IEM is not practicable with larger sample numbers and not suitable to differentiate the diverse virus variants. In contrast to our observations in Nigeria, Harper et al. (2004) detected more infections in Uganda by PCR than by IEM, when using BSV degenerate primers. The difference to our observations might be an indication for regionally differing diversities of bacilliform banana viruses coupled with the fact they used badna degenerate primer while we used BSV Onne primers. It was possible than more BSV isolates would have been detected using degenerate badnavirus primers that were not available when these experiments were done. Whether these primers could amplify all BSV variants has been questioned (Harper et al., 2004). There is thus the need to sequence BSV variants in Nigeria and Ghana, to determine their genomic variability and thus design suitable primers for their amplification.

CONCLUSION

In conclusion, our results underline the high variability of the badnavirus variants inducing leaf streak symptoms of banana. As a consequence, it has to be reconsidered in which way a virus indexing of banana cultivars has to be done in order to avoid a further world wide distribution of these viruses by the exchange of infected germplasm. Further on, it has to be reconsidered which methods have to be applied for a reliable virus screening during *in vitro* propagation and virus elimination programs. An open question also remains: how many species of badnavirus that infect *Musa* species exists? With many variants and species of BSV being found in different countries, how reliable is our recent degenerate badna primers?

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REFERENCES

Adomako D, Lesemann DE, Paul HL (1983). Improved methods for the purification and detection of cacao swollen shoot virus. Ann. Appl. Biol. 103: 109-116.

Agindotan BO, Thottappilly G, Uwaifo A, Winter S (2003). Production of monoclonal and polyclonal antibodies against a Nig. isolate of *Banana streak virus*. Afr. J. Biotechnol. Vol. 2 (7): 171–178.

Bouhida M, Lockhart BEL, Olszewski NE (1993). An analysis of the complete sequence of a sugarcane bacilliform virus genome infectious to banana and rice. J. Gen. Virol. 74: 15-22.

Braithwaite KS, Gambley CF, Smith GR (1996). Sequence variability in sugarcane bacilliform virus. Abstract PW11-1. Xth International Congress of Virology, Jerusalem, Israel, 11-16 August, 1996, 130.

Brunt AA, Kenten RH, Nixon HL (1964). Some properties of cacao swollen shoot virus from cocoa leaves. J. Gen. Microbiol. 36: 303-309.

Clark MF, Adams AN (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.

Dahal G, Pasberg-Gauhl C, Gauhl F, Thottappilly G, Hughes JdA (1998). Studies on a Nigerian isolate of banana streak badnavirus: II. Effect of intraplant variation on virus accumulation and reliability of diagnosis by ELISA. Ann. Appl. Biol. 132: 263-275.

Dahal G, Gauhl F, Pasberg-Gauhl C, Hughes JD,Thottappilly G, Lockhart BEL (1999). Evaluation of micropropagated plantain and banana (Musa spp.) for banana streak badnavirus incidence under field and screenhouse conditions in Nigeria. Ann. Appl. Biol. 134: 181-191.

Daniells JW, Geering AD, Bryde NJ, Thomas JE (2001). The effect of *Banana streak virus* on the growth and yield of dessert bananas in tropical Australia. Ann. Appl. Biol. 139: 51-60

Davis RI, Geering ADW, Thomas JE, Guna TG, Rahamma S (2000). First records of *Banana streak virus* on the Island of New Guinea. Aust. Plant Pathol. 29: 281.

Geering ADW, Pooggin MM, Olszewski NE, Lockhart BEL, Thomas JE (2005). Characterisation of banana streak Mysore virus and evidence that its DNA is integrated in the B Genome of cultivated Musa. Arch. Virol. 150: 787-796.

Geering ADW, Thomas JE (2002). Banana streak virus. Description no. 290, Association of Applied Biologists Descriptions of Plant Viruses Online (http://www.dpvweb.net/).

Geering ADW, Thomas JE (2002). Banana streak virus. aab Descriptions of Plant Viruses Nb. 390: 14+3 pp.

Geering ADW, McMichael LA, Dietzgen RG, Lockhart BEL (2001). Analysis of the distribution and structure of integrated *Banana streak virus* DNA in a range of Musa cultivars. Mol. Plant Pathol. 2: 207-213.

Geering ADW, McMichael LA, Dietzgen RG, Thomas JE (2000). Genetic diversity *among Banana streak virus* isolates from Australia. Phytopathol. 90: 921-927.

Hardy VG, Braithwaite KS, Gambly CF, Teakle DS, Hayward AC, Harding EM, Dale JL, Smith GR. (1996). Variation within sugarcane bacilliform badnavirus. Phytopathol. 86: 17. Supplement Abstr. 152A

Harper G, Dahal G, Thottappilly G, Hull R (1998). Detection of episomal banana streak Badnavirus by IC-PCR. J. Virol. Methods 79: 1-8.

Harper G, Hart D Moult, S Hull (2002). Detection of Banana streak virus in field samples of bananas from Uganda. Ann. Appl. Biol. 141: 247-257.

Harper G, Hart D, Moult S, Hull R (2004). *Banana streak virus* is very diverse in Uganda. Virus Res. 100: 51-56.

Harper G, Hart D, Moult S, Hull R, Geering A, Thomas J (2005). The diversity of Banana streak virus isolates in Uganda. Arch. Virol. 150 (12): 2407-2420.

Hull R, Geering A, Harper G, Lockhart BE, Schoelz JE (2004). Family Caulimoviridae. In: Virus Taxonomy, VIIIth Report of the International Committee on Taxonomy of Viruses (C M Fauquet, M A Mayo, J Maniloff, U Desselberger, LA Ball, edts.) . Elsevier/Academic Press London, 385-396.

Jaufeerally-Fakim Y, Khorugdharry A, Harper G (2006). Genetic variants of *Banana streak virus* in Mauritius. Virus Res. 115: 91-98.

Lockhart BEL (1986). Purification and serology of a bacilliform virus associated with a streak disease of banana. Phytopathol. 76: 995-999.

Lockhart BEL (1994). *Banana streak virus*: In Compendium of Tropical Diseases (Ploctz, et al., eds). St Paul MN, USA: APS Press 88pp.

Lockhart BEL, Autrey LJC (1988). Occurrence in sugarcane of a bacilliform virus related serologically *to banana streak virus*. Plant Dis. 72: 230-233.

Lockhart BEL, Olszewski NE (1993). Serological and genomic heterogeneity of banana streak Badnavirus: Implications for detection in Musa germplasm. In Breeding Banana and Plantain for Resistance to Diseases and Pests. Ed. J Genry Montpellier, France: INIBAP. pp. 105-113.

Lockhart BEL, Ndowora TC, Olszewski NE, Dahal G (1998). Studies on integration of banana streak Badnavirus sequences in Musa: Identification of episomally-expressible badnaviral integrants in Musa genotypes. In: *Banana streak virus*: A Unique Virus-Musa Interaction? Eds E A Frison and S E Sharrock. Proceedings of a workshop of the PROMUSA Virol. working group held in Montpellier, France, 19-21 January 1998.

Muller E, Sackey S (2004). Molecular variability analysis of five new complete cacao swollen shoot virus genomic sequences. Archives of Virol.

Ndowora TCR, Lockhart, BEL (2000). Improved serological method for detecting *banana streak virus*. Acta Horticult. 540: 377–388.

Thottappilly G, Dahal G, Lockhart BEL (1998). Studies on a Nigerian isolate of banana streak Badnavirus. I. Purification and enzyme-linked immunoassay. Ann. Appl. Biol. 132: 253-261.