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Production of Polyclonal Antibody Against an Isolate of Yam-infecting Badnavirus from Nigeria

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Abstract: Integrated viral sequences and high sequence variability among badnaviruses complicates the development of specific reliable molecular detection tests for yam-infecting badnaviruses. Thus Serological techniques are of notable importance for routine testing and monitoring of these viruses. The major limiting factor to the use of serological techniques is the limited availability of antibodies. Rabbit polyclonal antibody was produced against a purified preparation of a yam-infecting badnavirus from Nigeria. Antibody titre was determined by Protein-A sandwich (PAS) enzyme-linked immunosorbent assay (ELISA). The antibody produced had a titre of 1:1280 in PAS-ELISA and detected yam-infecting badnaviruses in infected yam leaves from Nigeria, Ghana, Benin and Togo. The suitability of the antibody for use in immunocapture polymerase chain reaction (IC-PCR) was evaluated. The antibody successfully trapped both *Dioscorea alata bacilliform virus* (DaBV) and *Dioscorea sansibarensis bacilliform virus* (DsBV) for IC-PCR detection. The antibody produced in this study will enhance certification of yam planting materials across West Africa and also facilitate the safe international movement of yam germplasm.

Key words: Yam-infecting badnaviruses, antibody, PAS-ELISA, ACP-ELISA, IC-PCR

INTRODUCTION

Yam is one of the most important staple starchy food crops for people in West Africa where approximately 93% of the world's yam is produced annually (F.A.O., 2008). Yam provides a major source of income for smallholders in this region (Nweke, F.I., 1991) and was simultaneously ranked first among 20 most important food and agricultural commodities (ranked by value) in Nigeria, Cote d'Ivoire, Ghana, Benin and Togo in 2007 (F.A.O., 2008). Although yam tubers can be stored longer than most other tropical fresh products, tuber yield and quality are adversely affected by virus disease infections (Seal, S. and E. Muller. 2007). The use of infected vegetative planting materials and uncontrolled exchange of germplasm by farmers through porous land borders have resulted in the presence of yam viruses in all yam growing areas of West Africa (Eni, A.O., J.d'A. Hughes and M.E.C. Rey., 2008;Eni, A.O., J.d'A. Hughes and M.E.C. Rey., 2010; Odu, B.O., J.d'A. Hughes, 1999; Hughes, J.d'A., L. Dongo, 1997; Seal, S. and E. Muller. 2007). Results of surveys of major yam producing agro-ecological zones in Ghana, Benin and Togo revealed that badnaviruses are an emerging viral threat in the yam system in West Africa being detected in 97.7% of 136 locations sampled (Eni, A.O., J.d'A. Hughes and M.E.C. Rey., 2008;Eni, A.O., J.d'A. Hughes and M.E.C. Rey., 2010).

Several species of badnaviruses have been reported to infect yams in West Africa and elsewhere around the world (Eni, A.O., J.d'A. Hughes and M.E.C. Rey., 2008; Kenyon, L., B.S.M. Lebas,2008; Seal, S. and E. Muller. 2007). High sequence variability among badnaviruses and the reported integration of badna-like sequences into host genomes complicates the development and use of molecular detection tests for badnaviruses and necessitates the use of immunocapture-polymerase chain reaction (IC-PCR) (Braithwaite, K.S., N.M. Egeskov, 1995; Geering, A.D.W., N.E. Olszewski, 2005; Harper, G., G. Dahal, 1999; Harper, G., 1999).

Immunocapture PCR favours the amplification of DNA contained within the trapped viral capsids, excluding DNA derived from other genomes, thus ensuring that only episomal virus sequences are amplified (Geering, A.D.W., L.A. McMichael, 2000; Harper, G., G. Dahal,1999).

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Serological tests such as ELISA are also very useful for routine diagnosis of badnaviruses (Hughes, J.d'A., L. Dongo, 1998). Both ELISA and IC-PCR require quality antibodies with broad reactivity to ensure detection of all or most strains of a virus. However, antibodies against yam viruses are scarce being available only in a few laboratories and not in large volumes. Antibodies against *yam mosaic virus* (YMV), genus *Potyvirus* are the only yam virus antibodies commercially available. This paper reports the production of polyclonal antibodies against an isolate of yam-infecting badnavirus from Nigeria.

MATERIALS AND METHODS

Virus Propagation:

Tubers from *Dioscorea alata* cv. Sagbe (TDr 92-2) infected with DBV were planted in an insect proof screen house (28-32°C) and used for virus isolation. Symptomatic leaf samples were tested by ELISA for yam-infecting badnaviruses, *Yam mosaic virus* (YMV, genus *Potyvirus*), and *Yam mild mosaic virus* (YMMV, genus *Potyvirus*) and *Cucumber mosaic virus* (CMV, genus *Cucumovirus*) since source plants may have mixed infections. ELISA tests were done using antibodies available at the International Institute of Tropical Agriculture (IITA) Ibadan. Source plants that were positive only to yam-infecting badnavirus were selected for the virus purification.

Virus Purification:

Badnavirus isolation followed a modification of the methods by Adomako *et al*. (1983). Infected yam leaves were ground in 600 ml of extraction buffer (0.1M phosphate buffer pH 7.2 containing 0.01M diethyldithiocarbamate (DIECA) and 0.5% (w/v) hemicellulase). The homogenate was incubated for 2.5 h at room temperature and then at 4°C overnight. After filtration through cheese cloth, the filtrate was centrifuged at 3,000 *g* for 20 min. To the supernatant, 6% (w/v) NaCl and 9% (w/v) polyethylene glycol (PEG) was added and stirred for 2.5 h at room temperature. The mixture was centrifuged at 2,500 *g* for 3 min and the precipitated virus preparation was re-suspended in 200 ml of re-suspension buffer I (0.05M phosphate buffer pH 7.0 containing 0.01 M DIECA, 0.2 M NaCl and 0.01 % (v/v) 2- mercaptoethanol). After overnight incubation at 4°C, the mixture was centrifuged at 8,000 *g* for 20 min, the supernatant was retained. The pellet was re-suspended in re-suspension buffer I, incubated at room temperature for 30 min and centrifuged at 8000 *g* for 20 min. The two supernatants were combined and 2 g of pre-washed celite (Fluka, Switzerland) was added and the mixture was filtered under vacuum through Whatman No. 1 filter paper to remove the celite. The filtrate was centrifuged at 6,000 *g* for 2 min to sediment remaining celite. The supernatant was then centrifuged at 785,000 *g* for 2.5 h. The pellet in each centrifuge tube was re-suspended in 0.5 ml of re-suspension buffer II (0.02 M citrate buffer pH 6.1) and allowed to re-suspend properly at 4°C overnight. The virus preparation was stored at -20°C before use.

Antibody Production:

The purified virus preparations were used as antigen for antiserum production in a 3-month old New Zealand white rabbit. Prior to antigen injection, the rabbit were injected with Ivomectrin (ICOMEC, USA) at the rate of 0.1 ml/kg of animal weight to rid the animal of any internal and/or external blood sucking parasites that may reduce the quantity of serum obtained after the experiment. The animal was allowed two weeks after Ivomectrin injection before commencement of antigen injection.

Antibody was prepared by immunizing the rabbit intramuscularly with a mixture of 500 µl of virus preparation and 500 µl of Complete Freund's Adjuvant emulsion. The immunisation was repeated two more times with Incomplete Freund's adjuvant replacing Complete Freund's adjuvant. The injections were alternated between the left and right thigh muscles. Test bleeds were taken from one of the ear veins of the rabbit seven days after the last immunization for titre determination. The serum was separated and clarified of blood cells by centrifugation.

Titration of Antibodies:

Protein-A antibody sandwich (PAS)-ELISA was used for antibody titration. PAS-ELISA followed the method described by Edwards and Cooper (1985) using serial dilutions of the produced antibody. The A_{405} for the substrate in each well was measured in a DYNEX MRX microplate reader after 1 h.

Determination of Antibody Reactivity and Suitability for Immunocapture-polymerase Chain Reaction (IC-PCR):

To assess the reactivity of the antibody for the detection of various isolates of yam-infecting badnaviruses, infected yam leaves collected from Nigeria, Ghana, Benin and Togo in our previous studies, were re-tested by PAS-ELISA using a 1:250 dilution of the prepared antibodies. Leaf sap from *Musa* sp. infected with *Banana streak virus* (BSV), genus *Badnavirus*, was also tested. The A_{405} for the substrate in each well was measured after 1 h.

Ten previously tested badnavirus infected yam leaves, including two samples each from the DsBV group and the DaBV subgroups I and II (Eni, A.O., 2008) were re-tested by IC-PCR. Two uninfected yam leaves were used as negative controls. Immunocapture followed the coating and trapping method by Clark and Adams (1977) using 1:250 dilution of the prepared antibody. PCR was carried out as previously described (Eni, A.O., J.d.A. Hughes, 2008) using Badna FP 5 -ATG CCI TTY GGI ITI AAR AAY GCI CC-3 and Badna RP 5 -CCA YTT RCA IAC ISC ICC CCA ICC-3 primers (Seal, S. and E. Muller. 2007, Seal, S. and E. Muller. 2007). Ten µL of IC-PCR products was analysed on a 1.5% agarose gel using TAE (40 mM Tris-acetate pH 8.3, 1 mM EDTA) at 100 V for 1 hour.

RESULTS AND DISCUSSION

Virus Propagation:

Puckering, crinkling and interveinal chlorosis were observed on leaves of *D. alata* cv. Sagbe two months after planting. Puckering and crinkling persisted and intensified while the chlorosis became less visible over time. Although DaBV is reported to be transmitted by mechanical inoculation and insect vector to several yam species (Odu, B.O., 2004; Phillips, S., R.W. Briddon, 1999), attempts to transmit the yam-infecting badnavirus isolate used for this study from infected yam leaves to herbaceous test plants by mechanical and vector transmission were unsuccessful. Mild mosaic symptoms were observed on leaves of *Nicotiana occidentalis* L. and *Nicotiana benthamiana* L. mechanically inoculated but subsequent transmission from these test plants were unsuccessful. Transmission experiments using *Planococcus citri* Risso*.,* known vector of DaBV (Odu, B.O., 2004; Phillips, S., R.W. Briddon, 1999) and three other mealybug species, *Planococcus solani* Ferris, *Dysmicoccus brevipis* Cockerell and *Ferrisia virgata* Cockerell*,* were also unsuccessful (data not shown). The virus was consequently purified from infected *D. alata* yam leaves due to the challenges outlined above.

Virus Purification:

The pellets resulting from the virus purification were tiny and cloudy on resuspension, possibly due to the celite used. Although DaBV coat protein band of 56 kDa (Phillips, S., R.W. Briddon, 1999) could not be visualized in SDS-PAGE gels, the purified preparations reacted positively (with high OD value) to heterologus DaBV antibodies (available at IITA, Ibadan) in PAS-ELISA, suggesting that the concentration of virus in the purified preparations may have been very low to be seen on the gel.

Titration of Antibody:

Antibody dilutions were considered as detecting if the mean absorbance values (at 405 nm=A_{405}) of diseased sap were twice or more than those of the healthy sap at that dilution (Thottappilly, G., G. Dahal, 1998). The titre of the produced antibody was 1:1,280 after 1 hr of substrate incubation in PAS-ELISA (Figure 5). This low antibody titre obtained may by due to the low yield of the virus preparation used for immunisation. Previous efforts to extract badnaviruses from leaves of infected host plant, encountered several problems and yields were reported to be low (Phillips, S., R.W. Briddon, 1999, Yang, I.C., G.J. Hafner, 2003).

Determination of Antibody Reactivity and Suitability for Immunocapture-polymerase Chain Reaction (IC-PCR):

The badnavirus antibody produced detected yam-infecting badnaviruses in infected yam leaves from Nigeria, Ghana, Benin and Togo. The antibody also detected BSV in infected *Musa* sp leaves (Table 1). In IC-PCR, nine of the infected yam leaves produced the expected PCR band size of 579 bp (Seal, S. and E. Muller. 2007), although the intensity of the bands varied. As expected, the negative control samples used, did not amplify (Figure 2).

These results further confirm that badnaviruses infecting yam are serologically related to each other and to other members of the *Badnavirus* genus. Phillips *et al.* (1999) reported serological relationships among badnaviruses infecting yam and among badnaviruses in general. In their study, DaBV was trapped and

decorated, in immunosorbent electron microscopy (ISEM), by antisera raised against Dioscorea *bulbifera bacilliform* virus (DbBV), genus *Badnavirus*, *Sugarcane bacilliform virus* (ScBV), *genus Badnavirus*, and *Banana streak virus* (BSV) *genus Badnavirus*. They also found that immunoglobulin (IgG) extracted from two DbBV antisera detected both DbBV and DaBV by ELISA. The unsuccessful amplification of one of the infected sample by IC-PCR may be due to leaf deterioration resulting from long term freezer storage of the leaf tissue used.

The detection of various isolates of yam-infecting badnaviruses from Nigeria, Ghana, Benin and Togo using the antibody produced in this study, and the trapping and subsequent detection of members of the DsBV and DaBV subgroups I and II using this antibody, indicates that the antibody is useful for detecting the various species of badnaviruses infecting yam across West Africa. Seal and Muller (Seal, S. and E. Muller. 2007) reported the use of a general badnavirus antibody for the detection of DsBV by ISEM and PAS-ELISA. Thus the antibody produced in this study maybe regarded as a general polyclonal antibody for the detection of several species/strains of badnaviruses infecting yam.

The effective use of the yam-infecting badnavirus polyclonal antibody produced for ELISA and IC-PCR confirms that the polyclonal antibody produced in this study is useful for reliable virus indexing for yam-infecting badnaviruses both for disease monitoring in field survey samples and for routine laboratory use by national agricultural research stations across West Africa for certification and research purposes.

Table 1: Detection of yam-infecting badnaviruses isolates in yam leaves from Nigeria, Ghana, Benin and Togo; and BSV in leaves of *Musa species* by Protein-A sandwich enzyme-linked immunosorbent assay (PAS-ELISA)

Country of origin	Yam species	Symptoms	PAS-ELISA A_{405}	Detection factor[a]
Nigeria	*D. alata*	Healthy	0.125	0.0
Ghana	*D. rotundata*	Vein clearing	0.438	3.5
Ghana	*D. alata*	Mottle	0.341	2.7
Togo	*D. alata*	Vein clearing	0.259	2.1
Togo	*D. alata*	Chlorosis	0.257	2.1
Benin	*D. alata*	Asymptomatic	0.630	5.0
Benin	*D. alata*	Puckering	0.436	3.5
Nigeria	*D. alata*	Puckering	0.460	3.7
Nigeria	*D. rotundata*	Chlorosis	0.286	2.3
Nigeria	*Musa species*	Healthy	0.146	0.0
Nigeria	*Musa species*	Chlorotic streak	0.375	2.6
Nigeria	*Musa species*	Chlorotic streak	0.336	2.3

[a] A_{405} of infected sap/A_{405} of healthy. Value >2 is considered positive

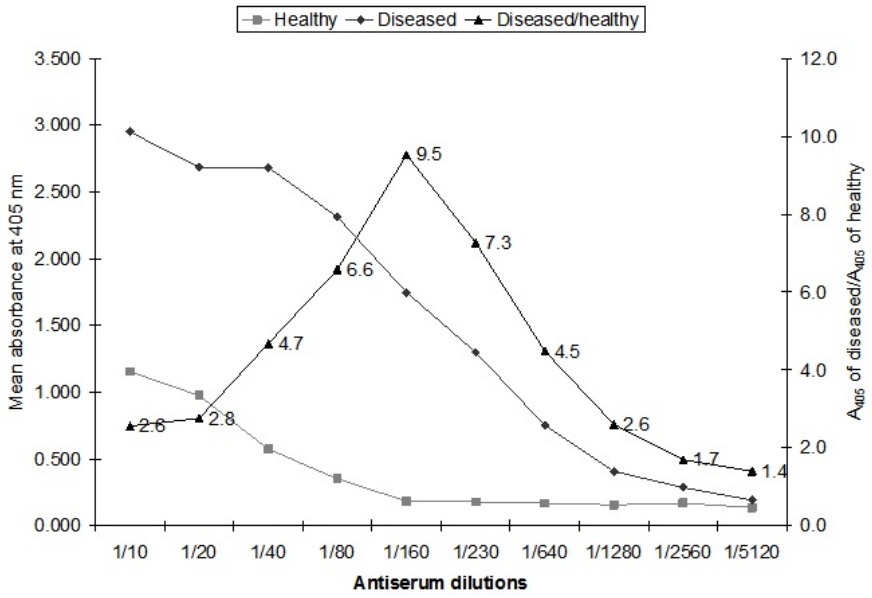

Fig. 1: Detection of yam-infecting badnaviruses in serially diluted infected and healthy sap (for titre determination) by Protein-A sandwich enzyme-linked immunosorbent assay (PAS-ELISA). A_{405} of infected sap/A_{405} of healthy sap ratio > 2 is considered positive.

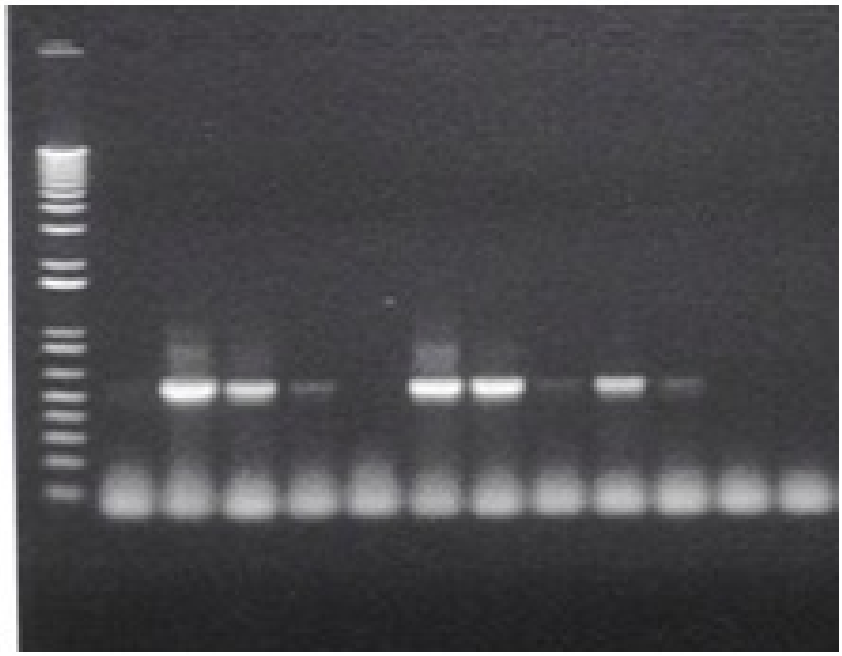

Fig.2: IC-PCR detection of yam-infecting badnavirus isolates from Nigeria, Ghana, Benin and Togo. Lanes 1-10 = isolates of yam-infecting badnaviruses, lanes 11 and 12 = negative controls

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