Practical Application of Virus Diagnostics in 'Cleaning-up' Yam and Cassava Germplasm for Distribution

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Abstract
The genebank at the International Institute of Tropical Agriculture (IITA) has 3,428 accessions of *Dioscorea* spp. and 2,385 of *Manihot esculenta*. These are maintained wherever possible as virus-tested *in vitro* plantlets. However, many of the accessions are virus-infected or are in the process of field rejuvenation, and therefore their health must be re-assured as they are taken into tissue culture for long-term conservation and subsequent multiplication for international germplasm distribution. The most important viruses infecting cassava in Africa, and prevalent in some of the genebank accessions, are the cassava mosaic geminiviruses, while *Dioscorea* spp. worldwide are infected by over 12 viruses, of which at least six are found in Africa, with *Yam mosaic virus* genus *Potyvirus* being the most prevalent. Diagnostic tests for the viruses infecting cassava and yams used in the clean-up of genebank accessions through meristem-tip culture include both protein-based and nucleic acid-based tests. The protein-based tests are serological tests, which are usually direct or indirect enzyme-linked immunosorbent assays, using monoclonal or polyclonal antisera. The nucleic acid based tests are a range of polymerase chain reaction (PCR) tests to detect both RNA and DNA viruses. These tests, with meristem tip culture and *in vitro* conservation, were carried out on cassava and yam plantlets for certification by the Nigerian Plant Quarantine Service. Out of the 45 *Dioscorea* spp. plantlets tested, a total of 27 were found to be infected with viruses infecting yams, while 124 cassava plantlets were infected out of the 422 tested. These results showed that both ELISA and PCR were effective in the production of virus free yam and cassava plantlets for healthy germplasm distribution.

Key words: *Dioscorea* spp., *Manihot esculenta*, virus diagnostics, germplasm, *in vitro*

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) and yam (*Dioscorea* spp. L.) are two of the most important staple food crops in Africa. World production of cassava in 2003 was over 189 million MT, with yields in sub-Saharan Africa (SSA) providing over 50% of the global production. Yam production in SSA was over 95% global production in 2003, with over 40

million MT yam tubers (FAOS TAT, 2009). The starch stored in the roots and tubers of both cassava and yam provides staple foods for over 200 million people in Africa alone. IITA's Genebank, with 3,428 accessions of *Dioscorea* spp. and 2,385 of *M. esculenta*, is an important repository of genetic material of yam and cassava in Africa. The genebank conserves the different accessions in a secure environment and makes the accessions available upon request. One of the key constraints to maintaining and disseminating the accessions is the large number of pests and diseases that can either infest and infect the material during field rejuvenation, or that can be carried through tissue culture (especially viruses) and therefore considerable effort has to be expended to clean up the accessions before they can be distributed. IITA follows international guidelines and standards for the distribution of germplasm and production of pest and disease-free and virus-tested plantlets requires substantial technical input.

Cassava is a woody perennial shrub cultivated for its starchy tuberous roots. The major pests of cassava in Africa are the cassava green mite (*Mononychellus tanajoa*), African root and tuber scale (*Stictococcus vayssierei*), the cassava mealybug (*Phenacoccus manihoti*) and the variegated grasshopper (*Zonocerus variegates).* The main diseases affectting cassava are cassava mosaic disease (CMD) caused by viruses in the genus *Begomovirus* (*African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV) and EACMV-Uganda variant), cassava bacterial blight (CBB; *Xanthomonas campestris* pv. *manihotis*), cassava anthracnose disease (CAD; *Colletotrichum gloesporiodes* f. sp. *manihotis*) and root rot (a complex of one or several of the following: *Botryodiplodia theobromae*, *Fusarium oxysporum*, *Fusarium solani*, *Sclerotium rolfsii*, *Armillaria mellea*) (Fokunang *et al.*, 2000). Pests and diseases, together with poor cultural practices, combine to cause yield losses that may be as high as 50% in Africa. The production of cassava is dependent on a supply of good quality stem cuttings. The multiplication rate of these vegetative planting materials is very low compared to grain crops, which are propagated by true seeds. In addition, cassava stem cuttings are bulky and highly perishable.

Important diseases of yam include yam anthracnose (*Colletotrichum gloeosporioides* (Penz.) Penz & Sacc.), tuber fungal

rot (*Fusarium* spp.), bacterial rots (*Erwinia* spp.) and several virus diseases (Emehute *et al.*, 1998). Virus-like symptoms including stunting, mosaic, necrosis, leaf distortion, mottling and banding on leaves of yams, and brown spots in the tubers have been observed on cultivated yams in the tropics (Harrison and Roberts, 1973; Brunt *et al.* 1990). Viruses reported to infect yams include *Dioscorea latent virus* (DLV), genus *Potexvirus, Dioscorea alata virus* (DAV), genus *Potyvirus, Cucumber mosaic virus* (CMV), genus *Cucumovirus, Dioscorea dumetorum virus* (DdV), genus *Potyvirus, Dioscorea bulbifera bacilliform virus* (DbBV), genus *Badnavirus, Dioscorea alata bacilliform virus* (DaBV), genus *Badnavirus* and *Yam mosaic virus* (YMV), genus *Potyvirus* (Brunt *et al.*, 1990; Hughes *et al.*, 1997; Odu *et al.*, 1999).

The objectives of this study were to ascertain the health status of genebank materials during the process of various virus eradication procedures from acquisition to the time of placing in the tissue culture, and to standardize the methods of cleaning up yam and cassava accessions.

MATERIALS AND METHODS

Cassava accessions undergoing field rejuvenation were planted in a ridged plot screenhouse with prophylactic insecticide (Cotchem, Springfield Agro Ltd., Singapore) sprays to prevent insect infestation and potential virus transmission from one plant to another. The cassava plants were evaluated for CMD incidence at 10 weeks after planting. The severity of the symptoms was quantified using the symptom severity ratings described by Terry (1975). Leaf samples were collected from actively growing plantlets in the screenhouse.

Plantlets of *Dioscorea* spp. being maintained *in vitro* were 'hardened' and planted in a containment room as part of the procedure at IITA for virus elimination from vegetatively propagated crops species. Most of the yam accessions brought in from field genebanks or which have been rejuvenated in the field are rapidly infected with viruses through vector transmission or may already have been infected. The yams were visually inspected for symptom expression after 12 weeks and indexed for virus infection using leaf samples collected from actively growing plants.

Indexing of the yam and cassava plants was by a combination of protein and nucleic acid-based procedures. Both enzyme-link immunosorbent assay

ELISA, in various formats, and Polymerase chain reaction PCR were used in this study, as well as the combination of serological and PCR methods in IC-PCR.

Enzyme-linked immunosorbent assays (ELISA).

Two main forms of ELISA were used for virus diagnostics in yam and cassava. While DLV, DAV, CMV, DdV, DbBV, DaBV and YMV are known to infect yams, only DAV, CMV, DaBV and YMV are know to commonly occur in West Africa and were thus the focus of the diagnostics. Similarly ACMV and EACMV were the targets of the cassava virus diagnostics.

Protein A-sandwich ELISA (PAS-ELISA) as described by Hughes and Thomas (1988) was used to detect DAV, DaBV and CMV in leaf samples of the established yam plants. This procedure uses protein A to trap and orientate trapping antibodies to the ELISA plate surface and protein A is conjugated to enzyme (alkaline phosphatase) to quantify the detecting antibodies that are trapped on the antigen. A sample was considered infected (positive) when the mean absorbance value at 405 nm (A_{405}) of the wells containing the sample was at least two times the absorbance of the wells containing the healthy control samples.

Serological detection of YMV, ACMV and EACMV was done with a triple antibody sandwich (TAS-) ELISA. The protocol described by Thottappilly *et al.* (1998) and Njukeng *et al.* (2002) was used to detect YMV in leaf samples, while the method of Thomas *et al.* (1986) was used for the detection of ACMV and EACMV in cassava leaf tissue samples. TAS-ELISA is used when monoclonal antibodies, raised through mouse myeloma cells, are employed in the ELISA procedure. As a result, a goat anti-mouse enzyme conjugate is used to quantify the antigen-trapped monoclonal antibodies. A sample was considered infected when the mean A_{405nm} of the wells containing the sample was equal to or greater than twice the A_{405nm} of the healthy, control sample.

PCR protocols

For the detection of CMD in cassava plants, the PCR method used was essentially as described by Ogbe *et al.* (2002). This procedure is a simple PCR protocol for use with DNA viruses in cassava. General geminivirus primers (Table 1) were used to detect infection by ACMV and EACMV in extracts of cassava leaf tissue.

In the case of viruses

infecting yams, immunocapture (IC)-PCR was used to detect YMV and DAV. As YMV and DAV are both RNA viruses, a reverse transcription step has to be incorporated. Thus IC- reverse transcriptase (RT)-PCR was used to detect these viruses, as described by Mumford and Seal (1997). IC-PCR was employed to detect the DNA virus, DaBV, in yam plant leaf samples after modifying the method of Mumford and Seal (1997) to exclude the reverse transcription step. The primers are listed in Table 1.

Table 1. List of primers used for detecting cassava and yam viruses.

Virus(es) targeted	Primer designation	Primer Sequences
Dioscorea alata virus (DAV), genus *Potyvirus*	DAVF	5'- GGC ACA CAT GCA AAT GAA RGC -3'
	DAVR	5'- CAC CAG TAG AGT GAA CAT AG -3'
Yam mosaic virus (YMV), genus *Potyvirus*	YMVF	5'- ATC CGG GAT GTG GAC AAT GA -3'
	YMVR	5'- TGG TCC TCC GCC ACA TCA AA -3'
Geminiviruses	UNIVERSA L2	5'- TGG ACY TTR CAW GGB CCT TCA CA -3'
African cassava mosaic virus (ACMV), *East African cassava mosaic virus* (EACMV) and EACMV-Uganda variant, genus *Begomovirus*	UNIVERSA L1	5'- TAA TAT TAC CKG WKG VCC SC -3'
Badnaviruses		
Dioscorea bulbifera bacilliform virus (DbBV),	BadnaRp	5'- CCA YTT RCA IAC ISC ICC CCA ICC -3'
Dioscorea alata bacilliform virus (DaBV)	BadnaFP	5'- ATG CCA TTY GGI ITI AAR AAY GCI CC -3'

RESULTS

Yam indexing

A total of 45 *in vitro* plantlets of *Dioscorea* spp. were evaluated for infection by YMV, DAV, DaBV and CMV. TAS-

ELISA detected YMV in 11 plantlets, while IC-RT-PCR identified an additional four plantlets that were infected by the virus (Table 2). A total of eight plantlets were infected with DaBV. PAS-ELISA was only able to detect the virus in one of the plantlets, while PCR detected the virus in seven additional plantlets. Four plantlets were confirmed by PAS-ELISA to be infected with CMV. DAV was not detected by either PAS-ELISA or IC-RT-PCR.

Table 2. Indexing results for 45 *in vitro*-derived plantlets of *Dioscorea* spp. tested for four viral infections using enzyme-linked immunosorbent assays (ELISA) and polymerase chain reaction (PCR).

Virus	Number of samples tested positive by ELISA	Additional number of samples tested positive by PCR
Yam mosaic virus (YMV), genus *Potyvirus*	11	+4
Dioscorea alata bacilliform virus (DaBV), genus *Badnavirus*	1	+7
Cucumber mosaic virus (CMV), genus *Cucumovirus*	4	
Dioscorea alata virus (DAV), genus *Potyvirus*	0	0
Total	16	+11

Cassava indexing

A total of 505 cassava plants were planted in the screenhouse for evaluation. Only 422 survived and were eventually scored for symptom severity (Table 3). Those 291 plants with a score of 1 had no symptoms. Using PCR, 14.08% of these symptomless plants were infected with CMD, while the remaining 250 plants tested were negative for the presence of cassava mosaic Geminiviruses.

CMD infection was detected by TAS-ELISA in only 80 out of the 131 plants that had symptom severity scores of 2, 3, and 4 (Table 3). Confirmatory testing by PCR identified a further three plants that were infected by CMD.

Table 3. Cassava germplasm indexed for the presence of African cassava mosaic virus (ACMV) and East African cassava mosaic virus (EACMV) using triple antibody sandwich enzyme-linked immunosorbent assays (TAS-ELISA) and Polymerase chain reaction (PCR).

[a] Score	Number of accessions	ELISA Positive	ELISA Negative	[b] Additional number of sample stested positive by PCR	Total number of infected plants
1	291	11	280	30	41
2	62	26	36	1	27
3	50	40	10	1	41
4	19	14	5	1	15
Total	422	91	331	33	124

[a]Symptom severity score as described by Terry (1975)
[b]Accessions that were negative by ELISA were tested and found to be infected by PCR

DISCUSSION

These results clearly show the difference in sensitivity and specificity between the protein-based ELISA virus detection methods and the nucleic acid based PCR methods. Detection of yam viruses using monoclonal and polyclonal antibodies was generally effective, but PCR was considerably more sensitive than ELISA for detecting the DNA virus, DaBV. In the case of virus screening in cassava, PCR can be reliably used to detect ACMV and EACMV using general geminivirus primers. However, no casual agent was identified for some of the plants exhibiting virus-like symptoms. These plants will be further monitored.

The implication of depending on visual observation for the diagnosis as previously done for the two crops is grave. The symptomless infections noticed in the lots could serve as potential foci of infection and establishment of the disease bearing in mind the presence of

vectors in the environment. There were 41 cassava plants that were actually infected while remaining symptomless.

In 'cleaning' up the cassava and yam accessions in IITA's Genebank, symptom expression, ELISA and PCR are used in various combinations to ensure that accessions destined for long-term storage or for international distribution can be certified free of virus, this is in line with the International Seed Testing Association regulation. This achievement has special significance for the safe movement of germplasm as to reduce risks of pathogen dissemination not only between continents or countries but even within national borders (Dixon *et al.*, 2001).

The use of sensitive, reliable and robust diagnostic methods for the detection of plant viruses is of utmost importance in certifying vegetatively propagated crops such as yam and cassava free of pathogen infection for international distribution/germplasm exchange. It is also important to determine the presence or absence of multiple infections in the two crops, especially in yams where the viruses are of different genus. Information of multiple virus infection is therefore needed to faction out strategies in eliminating them in tissue culture.

In the case of cassava, virus dissemination between fields and long distances is primarily through the use of infected stem cuttings as planting materials (Fauquet *et al.*, 1988), and secondarily by whitefly vector, while in yams the whole tubers and seed yams are the main sources of initial disease establishment (Odu *et al.*, 1999), complemented by vector such as *Aphis* spp. transmission. The use of virus tested tissue culture materials is therefore necessary in the production, distribution and establishment of disease free planting materials. This will also ensure the reduction in the incidence of these diseases in the growing areas of the respective crops.

Accurate disease diagnosis combined with sensitive and rapid detection of plant viruses is critical for the effective clean up of plant genetic resources available in the genebank. As both cassava and yam are distributed as *in vitro* plantlets, the main concern is to eliminate the plant viruses. Prior to the development of protein and nucleic acid-based detection methods, visual observation and herbaceous indicator plants were used to diagnose virus infection. The use of ELISA as a serological virus detection method using polyclonal antibodies improved the sensitivity of virus detection (Clark

and Adams, 1977). The advent of polymerase chain reaction (PCR) in the diagnosis of plant viruses has greatly improved the sensitivity of virus detection. The technique can also be combined with serological procedures in the form of immunocapture (IC)-PCR, combining the advantages of serology with the sensitivity of PCR to detect diseases (Nemchinov *et al.*, 1995; Wetzel *et al.*, 1992).

Without the use of PCR, these infected but seemingly symptomless plants would have been assumed to be free from virus infection, and they would have serve as source of infection to other virus-free plants in the field.

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