Production of Polyclonal Antibodies Against a Yam Isolate of Cucumber Mosaic Virus (Cmv)

[1,2,3]AO Eni, [1,4]Jd'A Hughes and [2]MEC Rey

[1]International Institute of Tropical Agriculture (IITA), Oyo Road, Ibadan, Nigeria
[2]School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg, South Africa
[3]Present Address: Department of Biological Sciences, Covenant University Km 10, Idiroko road, Canaanland, PMB 1023, Ota, Nigeria
[4]AVRDC – The World Vegetable Center, Shanhua, Taiwan

Abstract: *Cucumber mosaic virus* (CMV) genus *Cucumovirus* was recently detected in yam in Ghana, Togo and Benin bringing to six the total number of countries reporting CMV infection in yam worldwide. Two serotypes of CMV are distinguished and a specific antibody against the yam isolate of CMV is currently not available. Rabbit polyclonal antibodies were produced against purified preparations of a yam isolate of CMV from Nigeria. The antibody titre was determined by Protein-A sandwich (PAS) enzyme-linked immunosorbent assay (ELISA) and antigen-coated plate (ACP) ELISA. Antigen detection limit of the antibody was determined by PAS-ELISA using serial dilutions of infected sap. The CMV antiserum produced had a titre of 1:25,600 and 1:64,000 by PAS- and ACP-ELISA, respectively and a sap dilution end point of 1:160. The antibody detected homologous antigen in infected yam leaves from Ghana, Togo, Benin and Nigeria. The CMV polyclonal antibody produced in this study will enhance CMV monitoring and contribute to prevention of the spread of CMV infection which is spreading in yam.

Key words: Cucumber mosaic virus, Yam, Antibody, ELISA,

INTRODUCTION

Tubers of domesticated yam (*Dioscorea* spp.) are one of the major staple foods for millions of people in the tropical and sub-tropical regions of the world [17]. They are a source of carbohydrate, protein, amino acids, vitamins and minerals [4] and they can be stored longer than most other tropical fresh products. Yam production is adversely affected by virus disease infections which significantly reduce tuber yield and quality. Recent surveys in major yam producing agro-ecological zones in Ghana, Togo and Benin revealed for the first time, the occurrence of *Cucumber mosaic virus* (CMV) infection in yam in Ghana, Togo and Benin [7]. CMV has the widest host range of any known plant virus and is a potential cause of virus epidemics in many economically important crops [18]. Previous reports of CMV infection in yam have been only in three countries in the world namely Guadeloupe, Côte d'Ivoire and Nigeria [15,9,12], so the recent detection of CMV infection in yam in Ghana, Togo and Benin indicates a recent introduction and/or spread and needs to be monitored. Five of these six countries (Nigeria, Côte d'Ivoire, Ghana, Benin and Togo) account for over 90% of world yam production [8].

Swift and accurate diagnosis is vital for monitoring and control of plant viruses and for certification and breeding purposes. The use of symptomatology, either in the natural yam host or herbaceous indicator plant species, for diagnosis of yam viruses is unreliable due to the variability of symptoms caused by changes in environmental factors, differences in the yam cultivars or varieties of test plants and/or the strains of the virus(es). Symptomless CMV infections were reported in yam in Ghana, Togo and Benin and over 90% of CMV infections detected in these countries, occurred as mixed infections with *Dioscorea bacilliform virus* (DBV, genus *Badnavirus*), *Yam mosaic virus* (YMV, genus *Potyvirus*), and/or *Yam mild mosaic virus* (YMMV, genus *Potyvirus*) and thus further complicates the possibility of using visual symptoms for CMV identification [7]. Enzyme-linked immunosorbent assay (ELISA) is more routinely used for the diagnosis of yam viruses [13]. Although antibodies against CMV are readily available commercially, serological differences have been reported for CMV isolates [18,21] and a specific antibody against the yam isolate of CMV is currently not available. This paper reports the production of polyclonal antibodies against a yam isolate of CMV from Nigeria.

Corresponding Author: AO Eni, International Institute of Tropical Agriculture (IITA), Oyo Road, Ibadan, Nigeria
Email: angelaeni@gmail.com

MATERIALS AND METHODS

Virus propagation: Sap extract obtained by grinding CMV infected yam leaves in 0.1 M phosphate buffer pH 7.7, containing 10 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM L-cysteine at a ratio of 1:5 w/v (5 ml/g of leaf tissue) was mechanically inoculated to carborundum-dusted (600 mesh) upper surface of fully expanded cotyledons of one-week-old *Cucumis sativus* L. in an insect-proof screen house. Seven days post-inoculation (dpi), symptomatic leaves were tested by ELISA for CMV, DBV, YMV and YMMV as previously described by Eni *et al*. [6] using antibodies from the International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria. Test plants that were positive only to CMV were propagated on *C. sativus* for CMV purification.

Virus Purification: CMV purification was performed using the method described by Roossinck and White [20]. Freshly harvested CMV infected *C. sativus* leaves were homogenised in a mixture of buffer A (0.5 M sodium citrate, pH 7.0, 5 mM EDTA, 0.5% (v/v) thioglycolic acid) and 1ml of cold (4°C) chloroform for each gram of tissue. The homogenate was centrifuged at 15,000 *g* for 10 minutes at 4°C (Hermle Z232K**,** Hermle, Germany). The aqueous phase was filtered through cheese cloth, under-laid with 10 ml of cushion I (0.5 M sodium citrate, pH 7.0, 5 mM EDTA, 10% (w/v) sucrose) and centrifuged at 212,000 *g* for 1.5 h at 4°C in a Beckman L5-50B ultracentrifuge (Beckman Instrument Inc. California). The pellets were re-suspended in buffer B (5 mM sodium borate, pH 9.0, 5 mM EDTA, 2% (v/v) triton X-100), allowed to stir for 2 h at 4°C and centrifuged at 7500 *g* for 10 minutes at 4°C. The resulting supernatant was under-laid with 5 ml of cushion II (5 mM sodium borate, pH 9.0, 5 mM EDTA, 10% (w/v) sucrose) and centrifuged again for 212,000 *g* for 1.5 h at 4°C. The final pellets were re-suspended in buffer C (5 mM sodium borate, pH 9.0, 5 mM EDTA). The virus preparation was stored in buffer C at -20°C before use.

Using 5 as the extinction coefficient for CMV (10), the concentration of the purified virus preparation was calculated by spectrophotometry assuming $E^{0.1\%}_{260}$ = 5 = 1mg CMV/ml

Molecular weight determination: To determine the protein molecular weight of the purified virus preparation and to confirm its purity and the absence of contaminating protein before use for antibody production, the purified virus preparation were subjected to a discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using the protocol described by Laemmli [14]. A 12.5% resolving gel and a 4% stacking gel were used [23].

Low range protein molecular weight marker (Sigma-Aldrich Co., UK) was also loaded on the gel in a side lane for comparison with viral coat protein.

Antibody production: The purified CMV preparation was used as antigen for antiserum production in New Zealand white rabbit. Prior to antigen injection, the rabbit was injected with Ivomectrin (ICOMEC, USA) at the rate of 0.1 ml/kg of animal weight to rid it 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.

To elicit an immune response in the rabbit, four intramuscular injections of the purified virus preparation were administered over an eight-week period. The first three injections were given at two weeks intervals while the fourth one was given 28 days after the third injection. The injections were alternated between the left and right thigh muscles. The first injection consisted of 1 ml of purified virus preparation emulsified with 1 ml of Freund's complete adjuvant while subsequent injections of 1 ml purified virus were emulsified with 1 ml of incomplete Freund's adjuvant.

Test bleeds were taken from one of the ear veins of the rabbit two weeks after the second and third injections for titre determination. A final bleed was done two weeks after the last booster injection. After each bleeding, the collected blood was incubated in a glass beaker at 37°C for 1 h. The clot was ringed with a spatula and further incubated at 4°C overnight to allow the clot to shrink and the serum to completely separate. The serum was collected and centrifuged at 6000 *g* for 5 min to further clarify the serum of remaining red blood cells. The antibody was kept at 4°C before titre determination.

Titration of antibodies: Antigen coated plate (ACP) ELISA and PAS-ELISA were used for antibody titration. PAS-ELISA followed the method described by Edwards and Cooper [5]. An initial antibody dilution of 1:100 was made followed by double-fold serial dilution to 1:51,200. Antibody dilutions were made in phosphate buffered saline containing 0.05% v/v Tween-20 (PBS-T). Sap (200 µl) obtained by grinding CMV infected leaf tissues and healthy yam leaves in grinding buffer (PBS-T containing 0.5 mM polyvinyl pyrrolidone (PVP)-40 and 79.4 mM Na_2SO_3) were loaded in duplicate wells for each antibody dilution.

For ACP-ELISA, an initial antibody dilution of 1:500 followed by a two-fold serial dilution to 1:256,000. Antibody dilutions were made in conjugate buffer (half strength phosphate buffered saline (PBS) containing 0.05% (v/v) Tween-20, 0.02% (w/v) egg albumin, 0.005 mM PVP-40). Wells of microtitre plates

were coated with 100 µl of sap obtained by grinding CMV infected leaf tissues and healthy yam leaves in 0.05 M sodium carbonate buffer, pH 9.6 containing 0.5 mM PVP-40 and 79.4 mM Na_2SO_3. After a skimmed milk blocking step, 100 µl of each antibody dilution was loaded into duplicate wells pre-coated with diseased and healthy sap. Goat anti-rabbit alkaline phosphatase (Sigma, UK) diluted 1:40,000 in conjugate buffer were added to each well, followed by addition of 200 µl p-nitrophenylphosphate substrate (pNPP) (1 mg ml^{-1} in 10% diethanolamine, pH 9.8) into each of the wells to detect the antigen-antibody reactions.

Both for PAS-ELISA and ACP-ELISA, the A_{405} for the substrate in each well was measured in a DYNEX MRX microplate reader after 1 h.

Determination of antibody sensitivity/reactivity: The detection limit of the antibody was determined using serial dilutions of CMV infected sap. A 1:6,000 antibody dilution was used. Infected leaf tissues were ground in 10 volumes of grinding buffer and then diluted in a two-fold series to 1:640. Two hundred micro litres of each sap dilution was added to four wells of pre-coated ELISA plate and PAS-ELISA carried out as previously described by Edwards and Cooper [5]. The test for each virus was replicated four times. The A_{405} for the substrate in each well was measured in a DYNEX MRX microplate reader after 1 h.

To assess the reactivity of the antibodies for the detection of CMV isolates, CMV-infected yam leaves from Ghana, Togo, Benin and Nigeria were tested by PAS-ELISA using a 1:6,000 dilution of the prepared antibody. CMV infected leaves of *Nicotiana tabaccum* L. were also tested.

RESULTS AND DISCUSSION

Virus propagation/ purification: About 50% of the leaves of the mechanically inoculated *C. sativus* showed mosaic symptoms three dpi. The symptoms intensified progressively with over 70% leaf symptoms 7 dpi when the plants were harvested for virus purification. *Cucumis sativus* was used as a propagative host for CMV in this study, because mucilaginous substances contained in yam leaves are a problem to virus purification [25].

Clear glassy pellets were obtained after the final high speed centrifugation. The concentration of the purified virus preparation was 0.28 mg/ml using 5 as the extinction coefficient for CMV [10].

Molecular weight determination: The molecular weight of the purified CMV coat protein was estimated from the known molecular weight of the proteins contained in the low range protein molecular weight marker used. A single clear protein band of estimated size 29 KDa was observed in SDS-PAGE for the purified CMV preparation. This band size differs from the 24, 25 or 26 kDa previously reported for CMV coat protein[11,19]. This difference may be due to incomplete denaturation of the coat protein [2] or due to variation in the gel concentration. However, positive serological results were obtained when sap from previously tested CMV-infected leaf tissues from IITA were tested using the CMV antibody produced with this virus preparation.

Titration of antibodies: The titres of the first two test bleeds taken were low (result not shown). The titre of the third bleed taken after the forth injection was determined by PAS-ELISA and ACP-ELISA. Antibody dilutions were considered as detecting if the mean absorbance values of diseased sap at 405 nm (A_{405}) were twice or more than those of the healthy sap [24]. For PAS-ELISA, the A_{405} of the infected sap continued to be more than two times that of the healthy sap up to 1:25,600 dilutions (Figure 1). CMV detection by ACP-ELISA continued up to 1:64,000 dilutions (Figure 2). Peak detection factor (A_{405} of infected sap/A_{405} of healthy sap) for PAS-ELISA (4.8) was obtained at 1:3,200 (Figure 1) however, the close detection factor obtained at 1:6,400 dilution (4.3) informed the use of the antibody at 1:6,000 dilution for the antibody sensitivity/reactivity tests.

Determination of antibody sensitivity/reactivity: Sap dilution end point was used to determine the sensitivity of the antibody produced. CMV was detected from 1:10 to 1:160 sap dilutions (Table 1). The results obtained for the sap dilutions sensitivity of the CMV antibody produced in this study is similar to those obtained for the detection of YMV and YMMV in yam sap by Mumford and Seal (1997). The low sensitivity (1:160 for CMV-infected sap), can be attributed to low virus titre and/or inhibitory substances found in yam leaves[22,1].

The CMV antibody produced, detected CMV in infected yam leaves from Nigeria, Ghana, Togo and Benin (Table 2). CMV was detected both in *D. rotundata* and *D. alata*. CMV was also detected in infected *N. tabaccum* (Table 2). This broad reactivity of the CMV polyclonal antibody is useful in detecting all or most strains of the virus which has been reported to consist of two subgroups based on serological and nucleic acid properties[18,21]. The detection of CMV in both *D. rotundata* and *D. alata* from these countries also confirms that CMV infects *D. rotundata* and *D. alata* naturally possibly through one or more of its numerous aphid vector[3].
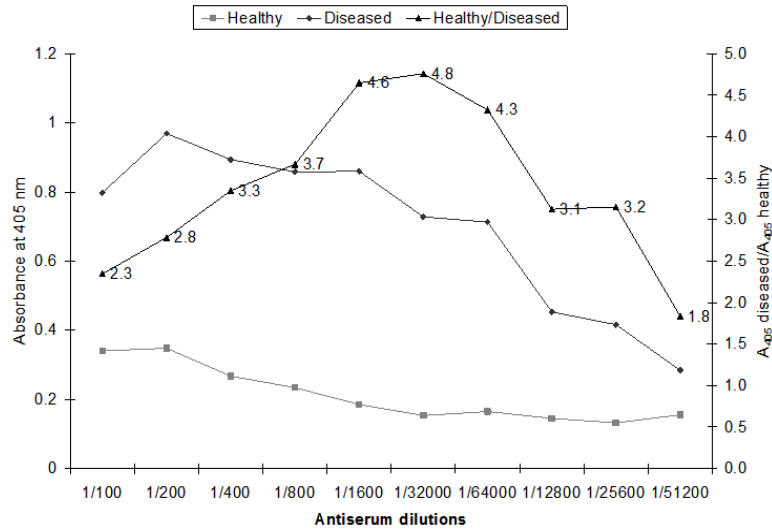
Fig. 1: Detection of *Cucumber mosaic virus* (CMV) in serially diluted infected and healthy sap (for titre determination) by Protein-A sandwich enzyme-linked immunosorbent assay (PAS-ELISA). A405 of infected sap/A_{405} of healthy sap ratio > 2 is considered positive.
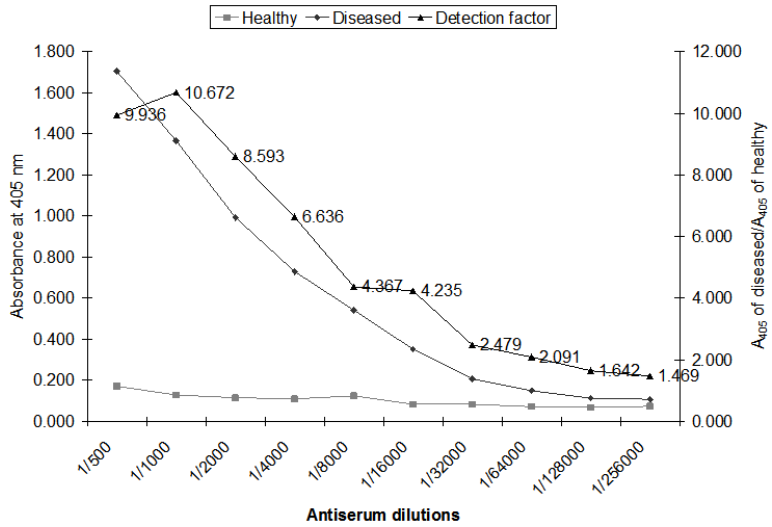

Fig. 2: Detection of *Cucumber mosaic virus* (CMV) in serially diluted infected and healthy sap (for antibody titre determination) by Antigen coated plate enzyme-linked immunosorbent assay (ACP-ELISA). A405 of infected sap/A405 of healthy sap ratio > 2 is considered positive.

Table 1: Detection of *Cucumber mosaic virus* (CMV) isolates in yam leaves and *N. tabaccum* from Ghana, Togo, Benin and Nigeria by Protein-A sandwich enzyme-linked immunosorbent assay (PAS-ELISA)

Country of origin	Yam species	symptom	PAS-ELISA A_{405}	Detection factor[a]
Nigeria	*D. alata*	Healthy	0.105	0.0
Ghana	*D. rotundata*	Asymptomatic	0.282	2.7
Ghana	*D. alata*	Asymptomatic	0.293	2.8
Togo	*D. alata*	Asymptomatic	0.241	2.3
Togo	*D. rotundata*	Puckering	0.349	3.3

Table 1: Continue

Benin	*D. alata*	Chlorosis, crinkling	0.245	2.3
Benin	*D. rotundata*	Mottle	0.408	3.9
Nigeria	*D. alata*	Chlorosis	0.330	3.1
Nigeria	*D. alata*	Asymptomatic	0.452	4.3
Nigeria	*N. tabaccum*	Healthy	0.112	0.0
Nigeria	*N. tabaccum*	Mosaic	1.766	15.8

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

Table 2: Sensitivity of *Cucumber mosaic virus* (CMV) antibody for the detection of CMV in dilution of infected yam sap by Protein-A sandwich enzyme-linked immunosorbent assay (PAS-ELISA).

Sap dilution	Mean absorbance at 405 nm	Detection factor[b]
Healthy	0.118	0.0
1/10	0.879	7.4
1/20	0.625	5.3
1/40	0.504	4.3
1/80	0.451	3.8
1/160	0.304	2.6
1/320	0.214	1.8
1/640	0.187	1.6

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

CMV infection is reported to be widespread [18] and causes virus epidemics and yield losses in many economically important crops. Its detection in yam in Ghana, Togo and Benin [7] indicate a spread of CMV infection in yam and this needs to be monitored. Yam is usually intercropped with melon, okra, potatoes, cowpea and other vegetables which are known CMV host species. These CMV infected crops may act as sources for vector transmission and will complicate efforts to control CMV infection in yam by use of virus-free planting materials. Naturally infected yam plants may also became sources of inoculum when used as planting materials the following year. To monitor the spread of CMV infection in yam and to prevent CMV accumulation in planting stock, farmers need to be adequately advised and health status of planting materials ensured by adequate yam virus indexing.

The CMV antibody produced in this study will improve the diagnostic capability for detecting and monitoring CMV in West Africa, contribute to preventing virus spread.

ACKNOWLEDGEMENTS

REFERENCES

1. Brunt, A., K. Crabtrea and A. Gibbs, 1990. Viruses of tropical plants. CAB International. Wallingford, UK.

2. Caillet-Boudin, M.L. and P. Lemay, 1986. Influence of the state of denaturation on the migration of adenovirus type 2 structural proteins in sodium dodecyl sulfate polyacrylamide gels. Electrophoresis., 7(7): 309-315.

3. Choi, S.K., J.K. Choi, W.M. Park and K.H. Ryu. 1999. RT-PCR detection and identification of three species of cucumovirus with a genus-specific single pair of primers. Journal of Virological Methods, 83: 67-73.

4. Degras, L., 1993. The Yam, a tropical root crop. The Macmillan Press, London, UK.

5. Edwards, M.L. and J.I. Cooper, 1985. Plant virus detection using a new form of indirect ELISA. Journal of Virological Methods, 11: 309-319.

6. Eni, A.O., J.d'A. Hughes and M.E.C. Rey, 2008. Survey of the incidence and distribution of five viruses infecting yam in the major yam producing zones in Benin. Annals of Applied Biology, 153(2): 223-232.

7. Eni, A.O., P. Lava Kumar, R. Asiedu, O.J. Alabi, R.A. Naidu, J.d'A. Hughes and M.E.C. Rey, 2008. First report of cucumber mosaic virus in yams (*Dioscorea* spp.) in Ghana, Togo, and Republic of Benin in West Africa. Plant Disease., 92: 833.

8. F.A.O., 2007. Food and Agricultural Organisation of the United Nations. FAO Statistics 2008. FAO, Rome. http://faostat.fao.org/

9. Fauquet, C. and J-C. Thouvenel., 1987. *In*: Plant Viral Diseases in the Ivory Coast, Documentations Techniques n°. 46, Editions de l'Orstom, Paris, pp: 29.

10. Francki, R.I.B., J.W. Randles, T.C. Chambers and S.B. Wilson, 1966. Some properties of purified cucumber mosaic virus (Q strain). Virology, 28: 720-741.

11. Haq, Q.M.R., B.P. Singh and K.M, Srivastava, 1996. Biological, serological and molecular characterization of a cucumber mosaic virus isolate from India. Plant Pathology, 45(5): 823–828.

12. Hughes, J.d'A., L. Dongo and G.I. Atiri, 1997. Viruses infecting cultivated yams (*Dioscorea alata* and *D. rotundata*) in Nigeria. Phytopathology, 87: 545.

13. Hughes, J.d'A., L. Dongo and S.Y.C. Ng, 1998. Diagnosis of yam viruses. Tropical Agriculture Trinidad, 75: 45-48.

14. Laemmli, U.K., 1970. Cleavage of structural proteins during assembly of the head of Bacteriophage T4. Nature., 227: 680-685.

15. Migliori, A. and B. Cadilhac, 1976. Contribution to the study of a virus disease of yam *Dioscorea trifida* in Guadeloupe. Annales de Phytopathologie, 8: 73–78.

16. Mumford, R.A. And S.E. Seal, 1997. Rapid single-tube immunocarpture RT-PCR for the detection of two yam potyviruses. Journal of Virology Methods, 69: 73-79.

17. Nweke, F.I., B.O. Ugwu, C.L.A. Asadu and P. Ay, 1991. Production costs in the yam-based cropping systems of south-western Nigeria. Resource and Crop Management Division. Research Monograph No 6, IITA Ibadan. pp: 29.

18. Palukaitis, P., M.J. Roossinck, R.G. Dietzgen and R.I.B. Francki, 1992. Cucumber mosaic virus. Advances in Virus Research, 41: 281-348.

19. Raj, S.K., A. Srivastava, G. Chandra and B.P. Singh, 2002. Characterization of cucumber mosaic virus isolate infecting *Gladiolus* cultivars and comparative evaluation of serological and molecular methods for sensitive diagnosis. Current Science, 83(9): 1132-1137.

20. Roossinck, M.J. and P.S. White, 1998. Cucumovirus isolation and RNA extraction. In: Plant Virology Protocols. G. D. Foster and S. C. Taylor, eds. Humana Press, Totowa, NJ, U.S.A., pp: 189-196.

21. Roossinck, M.J., L. Zhang and K.H. Hellwald, 1999. Rearrangements in the 5' Nontranslated Region and Phylogenetic Analyses of Cucumber Mosaic Virus RNA 3 Indicate Radial Evolution of Three Subgroups. Journal of Virology, 73(8): 6752-6758.

22. Rossel, H.W. and G. Thottappilly, 1985. Virus diseases of important food crop in Tropical Africa. International Institute of Tropical Agriculture, Ibadan Nigeria.

23. Sambrook, J., E. Fritsch and Maniatis, 1989. Molecular cloning: A laboratory manual 2nd edition. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

24. Thottappilly, G., G. Dahal and B.E.L. Lockhart, 1998. Studies on a Nigerian isolate of *Banana streak badnavirus* I. Purification and enzyme-linked immunoassay. Annals of Applied Biology, 132: 253-261.

25. Thouvenel, J.C. and C. Fauquet, 1979. Yam mosaic, a potyvirus infecting *Dioscorea cayenensis* in the Ivory Coast. Annals of Applied Biology, 93: 279-283.