Characteristics of a Cowpea Chlorotic Mottle Virus Isolate from Nigeria

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ABSTRACT

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Cowpea chlorotic mottle virus (CCMV, bromovirus group) was isolated from *Desmodium heterocarpon* and *Clitoria ternatea* plants growing in experimental plots at the International Institute of Tropical Agriculture, Ibadan, Nigeria. It was identified by serology, particle morphology, pH sensitivity, and analyses of viral coat protein and nucleic acids. In reciprocal agar-gel diffusion tests, the CCMV-Nigerian isolate and the type CCMV strain were found to be serologically distinct. This is the first report of the occurrence of a virus resembling CCMV outside of the United States and Costa Rica.

Additional keywords: CCMV serotypes, coat protein, genomic RNAs

Desmodium heterocarpon (L.) DC. and several other legumes, including *Clitoria ternatea* L., are under evaluation at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, for possible use as ground covers. During the past 3–4 yr, several *D. heterocarpon* and *C. ternatea* plants at IITA experimental plots exhibited brilliant yellow mosaic symptoms. Upon sap inoculation to cowpea (*Vigna unguiculata* (L.) Walp. 'IT-82E-10') and soybean (*Glycine max* (L.) Merr., several IITA breeding lines), a virus was readily transmitted from *D. heterocarpon* leaves that produced yellow mosaic symptoms. No reaction was observed, however, with an extract from the symptomatic *D. heterocarpon* leaves in agar-gel diffusion tests against antisera to several legume viruses that occur in Nigeria. A positive reaction was obtained with an antiserum to cowpea chlorotic mottle virus (CCMV, bromovirus group).

CCMV has only been reported from the United States and Costa Rica (1,2,8,9). It was first isolated from bean (*Phaseolus vulgaris* L.) in 1950 by Zaumeyer and Thomas in Illinois (14). A cowpea isolate recovered in 1962 in Georgia is considered the type CCMV strain (6,8). The natural hosts of CCMV are bean, cowpea, soybean, and *D. laevigatum* L. (2,6,9,12). CCMV is transmitted by leaf beetles (Coleoptera) and by sap inoculation (1,4,8). The virus is not carried through seed or by pollen (6,8).

We report here that the virus isolated from *D. heterocarpon* and *C. ternatea* in Nigeria shares many properties with CCMV but is antigenically distinct from the type strain. It is proposed to name this virus isolate CCMV-Nigerian (CCMV-N).

MATERIALS AND METHODS

The original isolate recovered from the field-infected *D. heterocarpon* plants was maintained by regular transfers to cowpea cv. IT-82E-10 by sap inoculation.

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Leaves were homogenized in 10 mM sodium acetate/acetic acid buffer, pH 5.0, and the extract was rubbed on leaves of test seedlings previously dusted with Carborundum powder. Leaves were then rinsed with water, and the plants were maintained in an insect-proof screenhouse for observation. For host range studies, extracts prepared from infected cowpea leaves were inoculated to various test plants.

For purification of CCMV-N virions, systemically infected cowpea leaves were homogenized (1 g/2 ml) in 100 mM sodium acetate/acetic acid buffer, pH 5.0. Ice-cold chloroform (final concn. 50%) was added, and the mixture was stirred for 5 min. The resulting emulsion was separated by centrifugation (10,000 *g* for 10 min), and virus from the aqueous phase precipitated with 8% polyethylene glycol and 20 mM NaCl. After resuspending the pellet in 50 mM acetate buffer, pH 5.0, and clarifying by low-speed centrifugation, the virus was sedimented (125,000 *g*, 2.5 hr, 5 C) through a 20% sucrose cushion. The resulting pellet was resuspended in 50 mM acetate buffer, pH 5.0, containing 0.025% sodium azide. The preparation was clarified by centrifugation at 10,000 *g* for 30 min and then submitted to rate-zonal sucrose density gradient centrifugation (7). The virions under the light-scattering zone were recovered and concentrated by ultracentrifugation. The pellets were resuspended in 50 mM acetate buffer, pH 5.0, containing azide; the suspension was clarified by low-speed centrifugation and stored at 5 C. Virus concentration was estimated spectrophotometrically with the extinction coefficient $E_{0.1\%}^{260nm} = 5.8$ (1,8).

The molecular weight of the viral coat protein was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5). In addition to molecular weight markers (Sigma Chemical Co., St. Louis, Missouri), coat proteins of southern bean mosaic virus (SBMV, sobemovirus group), rice yellow mottle virus (RYMV, sobemovirus group), and tobacco mosaic virus (TMV, tobamovirus group) were used as markers. Virion RNAs were isolated by the method of Wyatt and Kuhn (13) and analyzed with agarose gel electrophoresis (5,10).

Antiserum was produced in rabbits. Two intramuscular injections with incomplete adjuvant and two intravenous injections (approximately 4 mg total virus) were given, and the serum was collected 10 days after the last injection. Double-diffusion tests were done in 0.9% agarose in 50 mM acetate buffer, pH 5.0. Antisera to other plant viruses were from a collection being maintained at the IITA virology unit.

For electron microscopy, leaf segments were crushed on a glass microscope slide in 0.1% (w/v) sodium sulfite in distilled water, and a drop of the extract was placed on a carbon-reinforced, Formvar-coated grid, dried, and then stained with 2% (w/v) uranyl acetate, pH 4.6. Purified virion samples were stained similarly. Samples were examined in a Philips EM 201-C electron microscope.

RESULTS AND DISCUSSION

Host range and symptomatology. Field-infected *D. heterocarpon* and *C. ternatea* plants exhibited bright yellow mosaic symptoms, which varied from small circular spots or stipples to large patches, occasionally covering the entire leaflet (Fig. 1A and B).

In addition to *D. heterocarpon, C. ternatea,* cowpea, and soybean, CCMV-N was transmitted by sap inoculation to the following plant species. Leguminosae: *Centrosema pubescens* Benth., *P. vulgaris* cvs. Bataaf and Top Crop, *Rynchosia* sp., *Vigna subterranea* (L.) Verdc.; Chenopodiaceae: *Chenopodium amaranticolor* Coste & A. Reynier; Solanaceae: *Datura stramonium* L., *Nicotiana benthamiana* Domin. In most of these species, including soybean (Fig. 1C) and cowpea cv. IT-82E-10 (Fig. 1D), systemic bright yellow mosaic symptoms developed. In *C. amaranticolor*, infection was localized in the form of small chlorotic spots (Fig. 1E), and *N. benthamiana* proved to be a symptomless host of CCMV-N.

Symptoms did not develop nor was CCMV-N detected by immunodiffusion test and back-inoculation on cowpea plants in any of the following inoculated species. Leguminosae: *Arachis hypogea* L., *Cassia occidentalis* L.; Cucurbitaceae: *Cucumis sativus* L.; Gramineae: *Zea mays* L.; Malvaceae: *Abelmoschus esculentus* (L.) Moench; Solanaceae: *Lycopersicon esculentum* Miller, *Physalis floridana* Rydb., *Nicotiana megalosiphon* Heurch & Muell.

Electron microscopy. Numerous isometric particles were evident in leaf dip preparations from cowpea and *D. heterocarpon*. In purified samples, virions possessed an average diameter of approximately 28 nm (Fig. 2); TMV was used as the internal size standard. The overall shape or structure of CCMV-N was similar to that of the type CCMV strain (6,8).

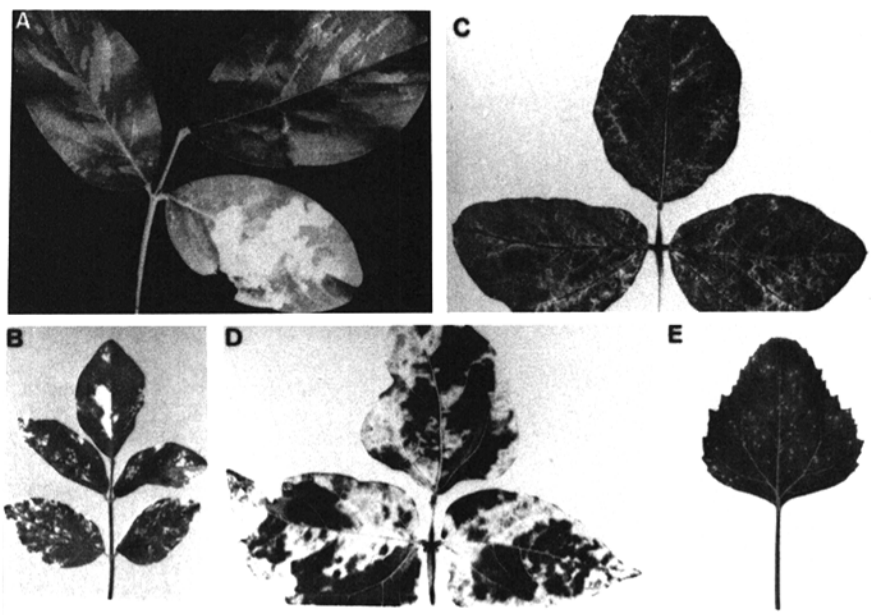

Fig. 1. Symptoms induced by the CCMV-Nigerian isolate on representative plant species. Naturally infected: **(A)** *Desmodium heterocarpon*, and **(B)** *Clitoria ternatea*. Infection resulting from sap inoculation: **(C)** *Glycine max*, **(D)** *Vigna unguiculata* 'IT-82E-10,' and **(E)** *Chenopodium amaranticolor*.

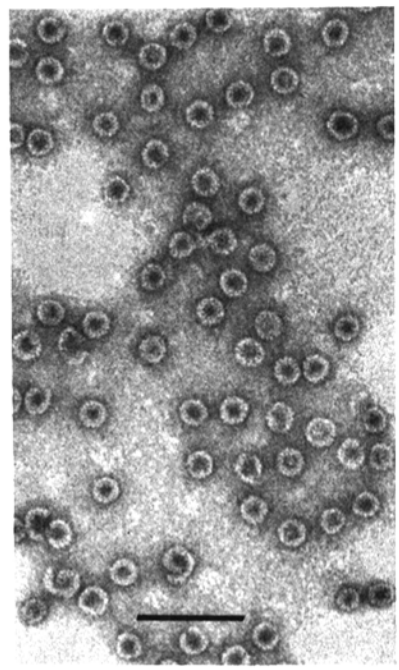

Fig. 2. Electron micrograph of purified virions of the CCMV-Nigerian isolate stained with uranyl acetate. Bar represents 100 nm.

Sensitivity to pH. CCMV-N virions maintained at pH 5.0 sedimented homogeneously at approximately 85S (Fig. 3A), similar to the type strain (1,8). When exposed to pH 7.0, most CCMV-N virions sedimented at about 78S, although some sedimented at 60S (Fig. 3B). The CCMV-N treated at pH 8.0 sedimented as a 60S entity (Fig. 3C). The type CCMV strain and other bromoviruses show similar instabilities at neutral or alkaline pH (1,8).

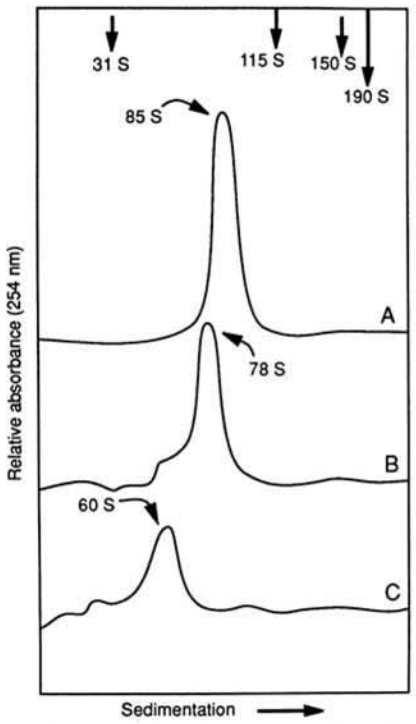

Fig. 3. The pH sensitivity of the CCMV-Nigerian isolate. Virions were incubated at 27 C for 1.5 hr in **(A)** 50 mM sodium acetate/acetic acid buffer, pH 5.0; **(B)** 50 mM sodium phosphate buffer, pH 7.0; or **(C)** 50 mM Tris-HCl buffer, pH 8.0, and then analyzed by rate-zonal sucrose density gradient centrifugation. The sedimentation markers used were TMV RNA (31S), SBMV virions (115S), maize dwarf mosaic virus virions (150S), and TMV virions (190S).

Molecular weight of the viral coat protein. CCMV-N virions had a single-coat protein with an apparent molecular weight of 20,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 12.5% gels (Fig. 4, lane b). Under similar conditions, the estimated molecular weight of SBMV or RYMV coat protein was approximately 30,000, while that of TMV was 17,000. The molecular weight of type CCMV strain is 19,600 (1,8).

Viral nucleic acids. CCMV-N virion nucleic acids were resolved into four distinctive species by agarose gel electrophoresis (Fig. 5). These nucleic acids were rapidly depolymerized by pancreatic ribonuclease at low and high salt concentrations, indicating that these were single-stranded RNAs (*data not shown*). The overall genomic composition of CCMV-N is comparable to that of the type CCMV strain and other bromoviruses (1,8).

Serology. Results of the agar-gel diffusion test showed (Fig. 6) that CCMV-N and the type CCMV strain were related but distinct. The precipitin line developed in the homologous reaction produced a strong spur with the heterologous antigen. Furthermore, the serological reaction was stronger in the homologous combination than in the heterologous combination. When antiserum to CCMV-N and its homologous antigen at 200 μg/ml was tested in the agar gel diffusion test, the antibody titer was found to be 1:2,048, whereas with the type CCMV strain, it was only 1:32, giving a serological differentiation index (SDI) of 6. Using CCMV type strain antiserum and the homologous antigen, the antibody titer was 1:256, whereas with CCMV-N, the antibody titer was 1:32, giving an SDI of 3.

Antisera to 44 different isometric viruses (belonging to 12 plant virus groups), including several that affect legumes in Nigeria (e.g., cowpea yellow mosaic virus, cowpea mottle virus, cucumber mosaic virus, and SBMV), gave no reaction when tested against purified CCMV-N or extracts from naturally infected *D. heterocarpon* or *C. ternatea* plants.

Four naturally occurring CCMV strains have so far been recognized based on symptomatology, relative infectivity, and serology (2,6,7,9,12). Walters and Dodd (12) isolated CCMV from *D. laevigatum* growing as a weed in Arkansas, and this isolate was serologically identical to the type CCMV strain. Consequently, it is different from CCMV-N. The CCMV-soybean strain (CCMV-S) differs from the type strain in symptom expression on cowpea and soybean and in specific infectivity, but the two are serologically identical (2,9). The bean yellow stipple virus (BYSV) strain of CCMV (CCMV-BYSV), reported from the United States and Central America, occurs primarily in bean (*P. vulgaris*) and can be distinguished serologically from the type strain on the basis of spur formation in double-diffusion tests (2,9). Similarly, another isolate from bean, CCMV-A, appears serologically different from the type strain and from CCMV-BYSV (2). Based upon the SDI values and reciprocally heterologous reactions (i.e., spur formation), CCMV-N and CCMV type strain are considered different serotypes (3). CCMV-N appears more closely related serologically to CCMV-BYSV and CCMV-A than to the type CCMV strain. In order to ascertain the precise relationship between the various CCMV strains, however, a direct and collective examination of their serological and other properties is necessary.

Although CCMV-N was transmitted

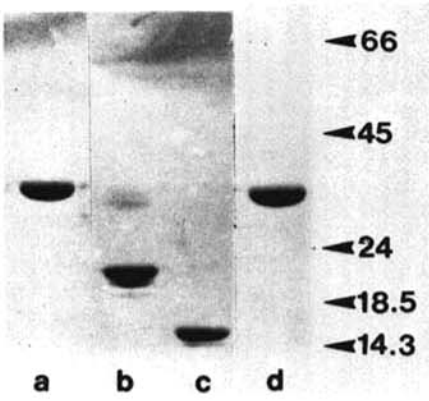

Fig. 4. Denaturing polyacrylamide gel electrophoresis of dissociated virions of a = SBMV, b = CCMV-Nigerian isolate, c = TMV, and d = rice yellow mottle virus. The positions of the molecular weight marker proteins are indicated on the right margin.

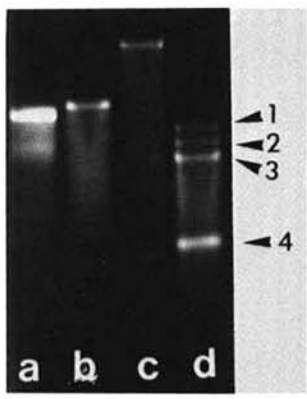

Fig. 5. Agarose gel electrophoresis of virion RNAs. a = SBMV, b = RYMV, c = a legume isolate of TMV, and d = CCMV-Nigerian isolate. The faster moving component in lane c is the mRNA of the TMV coat protein.

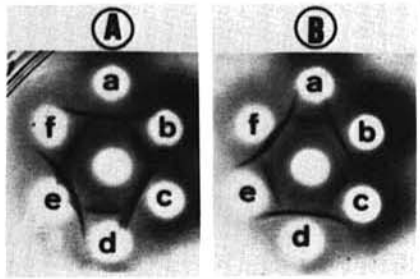

Fig. 6. Serological reactions of the CCMV-Nigerian (CCMV-N) isolate and the type CCMV strain with homologous and heterologous antisera. The central well in **(A)** was charged with antiserum of the type CCMV strain, whereas in **(B)** it was charged with antiserum to CCMV-N; both antisera were diluted 1:20. The peripheral wells contained a = type strain, 500 μg/ml; b = CCMV-N, 500 μg/ml; c = type strain, 200 μg/ml; d = CCMV-N, 200 μg/ml; e = type strain, 100 μg/ml; f = CCMV-N, 100 μg/ml. The charged plates were incubated at 25 C, stained with Coomassie brilliant blue, and then destained with water.

readily by sap inoculation to cowpea under our experimental conditions, extensive surveys and diagnostic tests conducted on samples received from various parts of Nigeria over the past several years indicated that it does not occur on cowpea or other cultivated legumes. Furthermore, this virus has not been observed on or recovered from weeds growing in the vicinity of the experimental plots at IITA; however, additional surveys and experiments are being planned to identify other field sources of CCMV-N. Although CCMV is transmitted by leaf-feeding beetles (*Cerotoma trifurcuta* Forster, *C. rufi-cornis* Olivier, *Diabrotica undecimpunc-tata howardi* Barber, and *D. balteata* LeConte), these insects are inefficient vectors of CCMV and of bromoviruses in general (4). The distribution and status of these insects as pests of legumes in Africa is not fully known (11). At present, CCMV-N does not pose any threat to cowpea, which is an important crop in the continent of Africa.

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