Detection of a Strain of Soybean Mosaic Virus Affecting Tropical Forage Species of *Centrosema*

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ABSTRACT

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A filamentous virus about 715 nm long was isolated from a plant of the tropical forage legume *Centrosema macrocarpum* affected by mosaic. Ultrathin sections of symptomatic leaf tissue showed pinwheel inclusions in the cytoplasm of infected cells. The virus was transmitted by mechanical means, by aphids, and through seed. Purified virus preparations had an absorbance ratio (A 260/280) of 1.13, contained a single protein of relative molecular weight 32,500, and had a 9-kb nucleic acid species. The virus was serologically indistinguishable from soybean mosaic virus and was related to bean common mosaic and watermelon mosaic-2 viruses. Based on the results of serological and pathogenicity tests, we conclude that the potyvirus isolated from *C. macrocarpum* is a strain of soybean mosaic virus.

The genus *Centrosema* contains species considered to be promising forage legumes for the acid, infertile soils of the tropics (24). More than 1,900 accessions of *Centrosema* spp. are currently available at the International Center of Tropical Agriculture (CIAT) for evaluation in tropical ecosystems (4). A mosaic and leaf distortion syndrome of apparent viral etiology has been observed in several *Centrosema* nurseries in Colombia.

Several filamentous and isometric viruses have been shown to infect *Centrosema* spp. The filamentous viruses reported include members of the potyvirus (1,13,17,18,27), carlavirus (10,11), and potexvirus (7,22,28,29) groups. The isometric viruses that infect *Centrosema* spp. include members of the comovirus (2,9,16,23), cucumovirus (19), and tymovirus (3) groups.

We undertook this investigation to characterize the causal agent of the mosaic and leaf distortion disease of *Centrosema* spp. in Colombia.

MATERIALS AND METHODS

Pathogen source. A plant of *C. macrocarpum* Bentham from Palmira (Valle), Colombia, with mosaic and foliar distortion symptoms was selected as the inoculum source.

Electron microscopy. Leaf extracts or partially purified preparations from infected plants were negatively stained in 2% uranyl acetate (pH 3.7) and examined for virus particles with a JEOL SX-100 electron microscope.

Mechanical inoculations. Infected leaf tissue was triturated in 0.1 M potassium phosphate buffer, pH 7.0 (1:10, w/v), and the extract was applied with a piece of sterile cheesecloth to the primary leaves of test plants. Inoculated plants were maintained in glasshouses at a maximum light intensity of 1,100 $\mu E \cdot m^{-2} \cdot s^{-1}$, an average temperature of 27 C, and 75% relative humidity.

Pathogenicity tests and host range. The following legume species were mechanically inoculated: *Arachis pintoi* Krapovickas & Gregory, *C. acutifolium* Bentham, *C. brasilianum* (L.) Bentham, *C. macrocarpum, C. pascuorum* Martius ex Bentham, *C. pubescens* Bentham, *Crotalaria* sp., *Desmodium barbatum* (L.) Bentham, *D. incanum* De Candolle, 16 cultivars of *Glycine max* (L.) Merr., *Macroptilium atropurpureum* (Moc. & Sesse ex DC.) Urb., *M. bracteatum* (Nees & Martius) Maréchal & Baudet, *M. erythroloma* (Bentham) Urban, *M. longepedunculatum* (Bentham) Urban, 21 cultivars of *Phaseolus vulgaris* L., and seven cultivars of *Vigna unguiculata* (L.) Walp. All test plants were examined for symptoms and indexed serologically or by electron microscopy 15–25 days after inoculation.

Insect transmission tests. Individuals of *Myzus persicae* (Sulz.) reared on red pepper plants (*Capsicum annuum* L.) were tested as vectors. Aphids were starved for 1–2 hr, then transferred to infected *C. macrocarpum* and Dubbele Witte bean plants for a 1-hr virus acquisition access period. The aphids were then transferred in groups of five to virus-free Dubbele Witte and Bountiful bean seedlings and allowed a 24-hr inoculation access period. Aphids were also transferred to healthy Dubbele Witte and Bountiful bean plants for 24-hr test feedings to serve as controls (none of these plants became infected in this study). After the inoculation period, aphids were killed with an insecticide.

Seed transmission tests. Seeds of four *C. pubescens* accessions (CIAT 438, 5144, 5634, and 15149) (1,950 seeds in all) were planted in trays (90–100 seeds per tray) inside an aphid-proof screenhouse. The emerged seedlings were assayed serologically by enzyme-linked immunosorbent assay (ELISA) 50 days after sowing. Fifty bean seeds harvested from Dubbele Witte plants systemically infected with the *Centrosema* virus were also planted, and the resulting seedlings were assayed by ELISA (1 mo after sowing) to test for seed transmission of the virus in *P. vulgaris*.

Virus purification. About 250 g of primary and first trifoliate leaves was harvested from systemically infected Dubbele Witte bean plants that had been inoculated 12 days earlier. Infected tissue was homogenized in a blender for 2 min in 500 ml of cold 0.5 M KPO_4 buffer, pH 7.7, containing 0.5 g of Na_2SO_3 and 1 mM Na-DIECA. The homogenized mixture was passed through cheesecloth and then stirred for 1 hr at 4 C with 8% *n*-butanol. The treated mixture was centrifuged at 4,080 *g* for 5 min, and the resulting supernatant was filtered through glass wool. The virus in the supernatant was precipitated with 4% polyethylene glycol 6000 (PEG) by stirring for 1 hr at 4 C and centrifugation at 8,000 *g* for 25 min. The concentrated virus was resuspended in 10 ml of 0.05 M KPO_4 buffer, pH 8.0, and maintained overnight at 4 C before the addition of 1% (v/v) Triton X-100. The suspension was stirred for 30 min at 4 C and then clarified for 5 min at 4,300 *g*. The supernatant was dialyzed overnight in distilled water before a second precipitation with 4 ml of 20% PEG in 0.5 M KPO_4 buffer, pH 8.0. This mixture was maintained at 4 C for 1 hr and then centrifuged at 18,800 *g* for 15 min. The precipitate was maintained in 0.05 M KPO_4 buffer, pH 8.0, kept overnight at 4 C, resuspended, and clarified for 10 min at 12,300 *g*.

The virus was further purified by equilibrium density gradient centrifugation (120,000 *g* for 300 min) in a preformed 20–35% (w/w) suspension of CsCl prepared in 0.05 M KPO_4 buffer, pH 8.0, in 5.5-ml centrifuge tubes. The virus band, which was located about 12 mm from the bottom of the CsCl gradient after centrifugation, was collected in a

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dropwise manner through a needle hole punched in the bottom of the tube. The material thus collected was diluted in 0.05 M KPO_4 buffer, pH 8.0, to a final volume of 5 ml. After a 10-min clarification at 4,000 *g*, the virus was concentrated by ultracentrifugation at 84,500 *g* for 120 min. The resulting pellet was resuspended overnight in 0.6 ml of 0.05 M KPO_4 buffer, pH 8.0, and given a 5-min clarification centrifugation at 12,300 *g*.

Spectrophotometry. Absorption spectra of purified virus preparations were obtained with a Beckman DU 50 spectrophotometer in the range of 360–240 nm. An extinction coefficient of 2.4 was used (20) to determine the probable concentration of the purified *Centrosema* virus.

Cytology. Leaf tissue of *C. macrocarpum* was prepared for thin sectioning by preliminary fixation with half-strength Karnovsky fixative (15) for 24 hr at 4 C, followed by a secondary fixation in 1% osmium tetroxide for 1 hr at room temperature. After dehydration in a graded series of alcohol and acetone, the fixed tissue was embedded in the low-viscosity epoxy resin medium described by Spurr (25). Sections were made with a diamond knife in an MT6000 Sorvall ultramicrotome.

Electrophoresis. Purified virus was analyzed in 10% polyacrylamide gels containing sodium dodecyl sulfate (SDS), as described by Weber and Osborn (30). Samples were dissociated by adding 2 vol of a solution containing 0.01 ml of electrophoresis $NaPO_4$ buffer, 0.25 ml of 10% SDS, 0.025 ml of 2-mercaptoethanol, and 0.25 ml of 60% sucrose and boiling the mixture for 1 min. Bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 20,000), cowpea mosaic virus coat proteins (M_r 22,000 and 42,000), and tobacco mosaic virus coat protein (M_r 17,500) were used as markers for molecular weight determinations.

The size of the viral nucleic acid was estimated by the addition of vanadyl ribonucleoside complex, 2 mg of proteinase K per milliliter, and 2% SDS for 1 min at 60 C. The extract was cooled to room temperature and run on a 1% agarose gel. The markers were red clover necrotic mosaic virus single-stranded RNA (ssRNA) (3.5 and 1.4 kb), ssRNA markers from Bethesda Research Laboratories (9.5, 7.5, 4.4, 2.4, and 1.4 kb), and cassava common mosaic virus RNA (6 kb).

Serology. An antiserum was prepared by injecting a New Zealand white rabbit with purified virus preparations standardized to a concentration of approximately 1 mg/ml. Four injections of 0.15 ml of the antigen, homogenized with an equal volume of Freund's complete (first injection) or incomplete (subsequent injections) adjuvant, were given at weekly intervals by the foot-pad technique of immunization (31).

Antisera to bean common mosaic, blackeye cowpea mosaic, soybean mosaic, and watermelon mosaic-2 viruses are maintained at the Virology Research Unit of CIAT. The serological test with passionfruit woodiness virus antiserum was performed by J. E. Thomas (Queensland Department of Primary Industries, Indooroopilly, Queensland, Australia).

Double-immunodiffusion, ELISA, and serologically specific electron microscopy (SSEM) tests were performed as described by Purcifull and Batchelor (21), Clark and Adams (6), and Derrick (8), respectively, using the antiserum obtained from the first bleeding.

Table 1. Reaction of selected bean, cowpea, and soybean cultivars to a filamentous virus isolated from mosaic-affected *Centrosema macrocarpum*

Cultivar	Reaction[a]
Bean	
Dubbele Witte	S
Stringless Green Refugee	L, S
Redlands Greenleaf C	L
Puregold Wax	L
Imuna	—
Redlands Greenleaf B	L
Great Northern 123	—
Sanilac	—
Michelite 61	L
Red Mexican 34	L, S
Pinto 114	—
Monroe	L, S
Great Northern 31	—
Red Mexican 35	—
Widusa	L, N, S
Black Turtle Soup	L, N
Jubila	L, N
Topcrop	—
Improved Tendergreen	L, N
Amanda	—
Bountiful	S
Cowpea	
293-476	—
2143 Peru 7	—
5006 ICA	—
Blackeye	S
Bush sitao	—
Monteria	—
Red Ripper	—
Soybean	
Clark	S
Rampage	S
Davis	—
York	—
Marshall	—
Ogden	—
Kwanggyo	—
Buffalo	—
ICA Lili	S
ICA line 109	S
ICA line 1211	S
ICA Mandarin	S
ICA Taroa	S
ICA Tunia	—
Improved Pelican	S
Williams	S

[a] S = systemic infection; L = local lesions in inoculated leaves; N = vein necrosis in inoculated leaves; — = no infection, as determined by absence of symptoms, electron microscopy, and serology.

RESULTS

Filamentous, flexuous particles about 715 nm long and 12 nm wide were observed in negatively stained leaf extracts of the mosaic-affected *C. macrocarpum* plant selected as the source of inoculum for this study. The virus was mechanically transmitted from *C. macrocarpum* to five *Centrosema* spp. and to susceptible *G. max*, *P. vulgaris*, and *V. unguiculata* cultivars (Table 1). None of the other manually inoculated legume species tested showed symptoms or was observed by electron microscopy or serology to harbor the virus.

Symptom expression in inoculated *Centrosema* spp. varied. Characteristic leaf distortion and mosaic symptoms (Fig. 1) were observed only in *C. macrocarpum*, about 45 days after inoculation. Less conspicuous mosaic and chlorosis symptoms were observed in *C. brasilianum*, *C. pubescens*, and *C. pascuorum* plants. Local chlorotic lesions appeared on the inoculated primary leaves of *C. pascuorum*.

The virus systemically infected nine of the 16 soybean cultivars tested (Table 1), inducing a yellow leaf veinclearing. Local and/or systemic symptoms were observed in 13 of the 21 bean cultivars

Fig. 1. Leaves of *Centrosema macrocarpum*, showing distortion and mosaic symptoms induced by a strain of soybean mosaic virus.

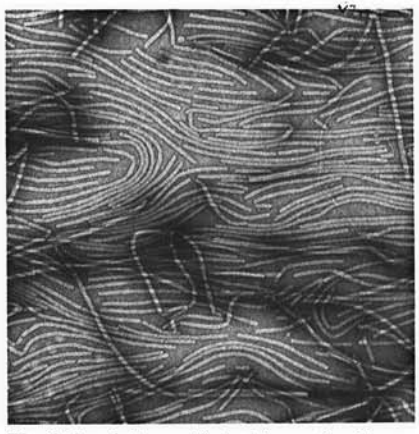

Fig. 2. Partially purified virions of a *Centrosema* strain of soybean mosaic virus negatively stained with 2% uranyl acetate. Scale bar represents 260 nm.

inoculated (Table 1). Bean cultivars Bountiful and Dubbele Witte were the most susceptible, showing severe mosaic and leaf malformation. All inoculated Dubbele Witte bean plants eventually died. Cultivars Stringless Green Refugee, Michelite 61, Red Mexican 34, and Monroe reacted with ring-shaped local lesions on the manually inoculated primary leaves. Inoculated primary leaves of cultivars Improved Tendergreen, Black Turtle Soup, Jubila, and Widusa developed vein necrosis. One Widusa test plant died from systemic necrosis. Cultivars Puregold Wax and Redlands Greenleaf B and C developed chlorotic local lesions on the inoculated primary leaves. Only one of the seven cowpea cultivars inoculated (Blackeye) became systemically infected and developed mosaic symptoms (Table 1).

The aphid *Myzus persicae* transmitted the virus from *C. macrocarpum* and Dubbele Witte bean to all test Dubbele Witte and Bountiful bean plants. The virus was transmitted via the seed of the four *C. pubescens* accessions (438, 5144, 5634, and 15149) and the bean cultivar (Dubbele Witte) tested, in percentages of 1.5 (6/400 seeds), 1.4 (11/770 seeds), 2.5 (10/400 seeds), 1.6 (6/380 seeds), and 6 (3/50 seeds), respectively.

The virus was isolated with a high degree of purity (Fig. 2) following the purification procedure outlined above. Virus yields were 0.5 mg per 100 g of bean tissue despite the noticeable virus aggregation problems observed throughout the purification procedure. Purified virus preparations exhibited an absorbance ratio (A 260/280) of 1.13. The SDS-polyacrylamide gel electrophoresis analysis of the virus coat protein revealed the presence of a single subunit of M_r 32,500. A single nucleic acid molecule of about 9 kb was detected in agarose gels.

The examination of ultrathin sections of mosaic-affected *C. macrocarpum* leaf tissue revealed the presence of cylindrical inclusions, consisting of pinwheels and scrolls (Fig. 3), in the cytoplasm of infected cells.

The virus was serologically indistinguishable from soybean mosaic virus (SMV) in reciprocal Ouchterlony, ELISA, and SSEM (1,000–1,100 particles per 1,000 μm^2) tests and was related to bean common mosaic and watermelon mosaic-2 viruses (weak serological reactions in Ouchterlony tests). The virus did not react with antiserum to blackeye cowpea mosaic virus or passionfruit woodiness virus in SSEM tests.

DISCUSSION

The virus isolated in this study from *C. macrocarpum* was determined to be a member of the potyvirus group based on its morphology, particle length, aphid transmissibility in a nonpersistent manner, and serological relationship with known members of the potyvirus group (14), the presence of pinwheel inclusions in infected plant cells, the molecular weight of its capsid protein subunit and nucleic acid genome, and the ultraviolet spectrum of the viral nucleoprotein. The close serological relationship of this virus to SMV and its pathogenic behavior in soybean and bean cultivars previously used to differentiate SMV strains (6,26) indicate that it belongs to the G1 group of SMV strains (6).

SMV is known to infect some 16 species in the Leguminosae (12). The high incidence of this virus in most *Centrosema* evaluation nurseries in geographically isolated regions of Colombia and the detection of the virus in the Caribbean area (F. J. Morales, *unpublished*) suggest that SMV is a major viral pathogen of this important tropical forage legume. SMV must be considered a potential constraint to the establishment of mixed grass-legume pastures in the tropics, particularly in Latin America and Africa, where several forage legume species are being collected and used for this purpose (24). Among the tropical forage legumes currently maintained by the Genetic Resources Unit of CIAT, the genera *Astragalus, Canavalia, Cassia, Crotalaria, Galactia, Lablab, Lespedeza,* and *Sesbania* are reported hosts of SMV (12). Finally, the demonstrated seed transmissibility of SMV in *Centrosema* spp. further supports the consideration of this virus as a pathogen of considerable economic importance. We propose the designation SMV-CE for this strain.

Fig. 3. Transmission electron micrograph of a thin section of *Centrosema macrocarpum* leaf tissue infected with a *Centrosema* strain of soybean mosaic virus, showing pinwheel inclusions in the cytoplasm of a parenchyma cell. Scale bar represents 330 nm.

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