Detection of a Potyvirus Related to Guineagrass Mosaic Virus Infecting *Brachiaria* spp. in South America

F. J. MORALES, Virologist, M. CASTAÑO, Research Associate, A. C. VELASCO, Research Assistant, and J. ARROYAVE, Electron Microscopist, Centro Internacional de Agricultura Tropical (CIAT), Apartado Aereo 6713, Cali, Colombia

ABSTRACT

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A filamentous virus approximately 775 nm long was isolated from variegated plants of the tropical forage grass *Brachiaria* spp. collected in Colombia and Brazil, South America. Ultrathin sections of symptomatic leaf tissue showed cylindrical inclusions (scrolls) in the cytoplasm of infected cells. The virus was transmitted mechanically to selected species of *Brachiaria, Panicum*, and *Paspalum* but not to maize, sorghum, or sugarcane. The virus was also transmitted from and to *B. brizantha* by the aphid *Myzus persicae* in a nonpersistent manner. The virus was not seedborne in *Brachiaria* spp. Purified virus preparations had an $A_{260/280}$ ratio of 1.30 and exhibited capsid protein heterogeneity (M_r 41, 34, 32, and 30 kDa) as determined by SDS-PAGE. The virus was closely related serologically to guineagrass mosaic virus from Africa, distantly related to watermelon mosaic virus-2, and not related to maize dwarf mosaic, sorghum mosaic, and sugarcane mosaic viruses. Results of our serological and pathogenicity tests suggest that the virus isolated from *Brachiaria* spp. is a strain of guineagrass mosaic potyvirus (GGMV). This would be the first report on the occurrence of GGMV in the Americas.

The genus *Brachiaria* includes several cultivated and promising species of forage grasses native to tropical Africa (2). The accidental or intentional introduction of *Brachiaria* germ plasm into the Americas probably started in the 17th century (21) and has continued uninterruptedly to date. It is estimated that *B. decumbens* Stapf, introduced in the late 1950s, occupied more than 15 million hectares in tropical America two decades later (21).

The main constraint to the adaptation of *Brachiaria* spp. in South America has been their susceptibility to insect pests (10). Recently, a variegation disorder was observed affecting *Brachiaria* spp. in different regions of Colombia and Brazil. This investigation was initiated to identify the causal agent, considering the economic importance of this forage grass and its contribution to the production of beef and dairy products in developing nations.

MATERIALS AND METHODS

Virus isolation and maintenance. Leaves of *B. brizantha* (Hochst.) Stapf affected by stippling, chlorotic streaks, and rhomboid lesions were collected in the eastern plains (Meta) of Colombia. Virus isolates were maintained by manual inoculation in *B. brizantha* cv. La Libertad under glasshouse conditions (maximum light intensity of 87 $\mu E \cdot m^{-2} \cdot s^{-1}$, 26 C, and 75% RH, average annual values) at CIAT, Palmira, Colombia. All mechanical transmission tests were done with foliar tissue obtained from virus-infected *Brachiaria* plants, homogenized in 0.1 M KPO_4 buffer, pH 7.0, (1:10, w/v). Leaf samples were also collected from *B. brizantha* plants showing similar symptoms near the localities of Santander de Quilichao, Cauca, Colombia, and Campo Grande, Mato Grosso do Sul, Brazil. The Brazilian samples were used only for serological assays.

Electron microscopy. Leaf extracts and purified virus preparations from infected test plants were negatively stained in 2% uranyl acetate, pH 3.7, for observation of virus particles with a JEOL SX-100 electron microscope. Leaf tissue of symptomatic *B. brizantha* plants was prepared for cytology as described earlier (14). Thin sections were cut with a diamond knife using an MT 6000 Sorvall ultramicrotome.

Pathogenicity tests. Manually inoculated *Brachiaria* spp. were *B. brizantha* (CIAT 6294, 6297, 6387, 6690, 6780, 16106, 16107, 16122, 16126, 16152, 16162, 16296, 16313, 16318, 16337, 16338, 16827, 16829, 16832, and 26646); *B. decumbens* (CIAT 606, 26185, 26186, and 26303); *B. dictyoneura* (Fig. & De Not.) Stapf (CIAT 6133, 16186, 16187, and 16508); *B. humidicola* (Rendle) Schweickerdt (CIAT 679, 682, 6705, 16181, and 16182); *B. jubata* (Fig. & De Not.) Stapf (CIAT 6409, 16195, and 16536); *B. platynota* (K. Schumann) Robyns (CIAT 26200 and 26340); and *B. ruziziensis* Germ. & Evrard (CIAT 641, 660, 6019, and 6692). Also inoculated were *Oryza sativa* L. 'CICA 8' and 'Bluebonnet 50'; *Panicum maximum* Jacq. (CIAT 673, 695, 6000, 6172, 6299, 6798, 6828, 6854, and 6856); *Paspalum conjugatum* Berg. and *P. dilatatum* Poiret.; *Pennisetum purpureum* Schumach. (CIAT 16076); *Saccharum officinarum* L. 'CO-281', 'CP-281', 'CP31-294', 'CP31-588', 'CP57-603', 'CC87639', and M 336 × PR 980; *Sorghum bicolor* (L.) Moench 'Atlas', 'IS-3071', 'MN 1056', 'MN 3071', 'Rio', and 'Serere I' and *S. halepense* (L.) Pers.; and *Zea mays* L. 'SA1', 'SA2', 'SA3', 'SA4', and 'SA6'. All test plants were serologically assayed 15–20 days after inoculation.

Insect transmission tests. Individuals (late nymphal stages) of *Myzus persicae* (Sulzer) reared on pepper (*Capsicum* sp.) were tested as potential vectors. Aphids were starved for 4 hr before being transferred to virus-infected *B. brizantha* plants. After a 5-min acquisition access period, aphids were transferred in groups of five to each of six virus-free, 15-day-old seedlings of *B. brizantha* (CIAT 26646). Three *Sorghum bicolor* cv. Rio plants and three *Saccharum officinarum* plants were also aphid-inoculated in the same manner. Following a 24-hr inoculation access period on all test plants, aphids were killed with an insecticide.

Seed transmission tests. A total of 340 *B. brizantha* and 660 *B. decumbens* mature seeds harvested from diseased plants grown under field conditions were homogenized in groups of 20 to be tested by DAS-ELISA. Approximately 100 seeds harvested from variegated *B. brizantha* plants were also sowed for visual and serological examination of seedlings.

Virus purification. Systemically infected tissue of *B. brizantha* CIAT 26646 was homogenized 45 days after manual inoculation in 2 ml of cold 0.5 M KPO_4 buffer, pH 7.6, containing 0.5 g of Na_2SO_3 per gram of infected tissue. The mixture was further homogenized in the presence of chloroform and carbon tetrachloride, using 0.5 ml of each solvent per gram of tissue. After centrifugation at

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4,080 *g* for 5 min, the supernatant was filtered through glass wool and treated with 6% PEG, stirring for 1 hr at 5 C. The virus was precipitated by centrifugation at 8,000 *g* for 20 min and then resuspended in 0.25 M KPO_4, pH 7.5, using 5 ml of buffer per 100 g of infected plant tissue. Following a clarification at 12,100 *g* for 5 min, the virus was treated again by adding 2 ml of 20% PEG in 0.25 M KPO_4 buffer, pH 7.5, per each 5 ml of the virus suspension. Following a 1-hr incubation period at 4 C, the virus was precipitated again at 12,100 *g* for 10 min, then resuspended in 1 ml of 0.25 M KPO_4 buffer, pH 7.5, per 100 g of infected tissue. The partially purified virus preparations were layered onto preformed 26–40% (w/v) CsCl suspensions prepared in 0.05 M KPO_4 buffer, pH 8.0. After centrifugation at 120,000 *g* for 5 hr, the virus bands located approximately 14 mm from the bottom of 5.5-ml centrifuge tubes were collected with a syringe and diluted in 0.2 ml of 0.25 M KPO_4 buffer, pH 7.5, to a final volume of 10 ml. The virus was concentrated by centrifugation at 84,000 *g* for 2 hr and finally resuspended in 0.25 ml of 0.05 M KPO_4 buffer, pH 7.5, per 100 g of the original amount of infected plant tissue.

The ultraviolet absorption spectrum of the purified virus was obtained with a Beckman DU 50 spectrophotometer in the 240- to 360-nm range. An extinction coefficient of 2.4 (7) was used to calculate virus concentration.

Electrophoresis. Purified virus preparations were analyzed in 10% polyacrylamide gels containing SDS as described by Weber and Osborn (23). Virus samples were prepared for electrophoresis as described earlier (13), using bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) as markers for molecular weight determinations.

Serology. An antiserum to the *Brachiaria* virus was prepared as described earlier (13). Antisera to maize dwarf mosaic virus (PVAS-55b), sorghum mosaic virus (PVAS-323a), and sugarcane mosaic virus (PVAS-115) were obtained from the American Type Culture Collection, Rockville, Maryland. Antisera to guineagrass mosaic potyvirus (JTC-185, BK-203, and BK-215) were kindly provided by Jean Dubern, LPRC/ORSTOM, Montpellier, France. A commercially available monoclonal antibody specific to the potyvirus group (Agdia, Inc., Elkhart, IN) was also used in this investigation. Four antisera to watermelon mosaic virus-2 (19, 202/1134, 868, and 1125) were obtained from D. E. Purcifull, University of Florida, Gainesville.

Double immunodiffusion, DAS-ELISA, plate-trapped antigen (PTA) ELISA, and serologically specific electron microscopy (SSEM) were conducted as described by Purcifull and Batchelor (17), Clark and Adams (3), Mowat and Dawson (15), and Derrick (4), respectively.

RESULTS

The causal agent was manually transmitted to virus-free test plants of *B. brizantha*, inducing eye-shaped lesions (Fig. 1A) and a variegation (Fig. 1B) characteristic of the original symptoms observed on *Brachiaria* spp. under field conditions.

The electron microscopic examination of *B. brizantha* leaf extracts obtained from both naturally and artificially infected plants demonstrated the presence of filamentous virus particles measuring an average (50 particles) of 775 nm in length but with a predominant class (21 particles) 783–803 nm long.

The cytological study of infected *B. brizantha* leaf cells showed the presence of cylindrical inclusions, mainly "scrolls" (Fig. 2A) but also "tubes" in longitudinal sections (Fig. 2B). No laminated aggregates were observed despite a thorough examination of different infected tissue samples.

Of 41 accessions representing seven *Brachiaria* spp. artificially inoculated in this study, only two accessions of *B. platynota* (26340 and 26343) could not be infected when mature plants were cut down to a height of approximately 10 cm to obtain smaller, actively growing leaves suitable for virus inoculation. We were unable to obtain seed of these accessions for seedling inoculation, a procedure that had resulted in the infection of other *Brachiaria* spp. that could not be infected in the adult plant stage by mechanical means. With the exception of *Paspalum conjugatum* and *Panicum maximum* CIAT 6299 (K-187), which were susceptible to the *Brachiaria* virus, the remaining artificially inoculated grass species were resistant and serologically negative for the presence of the virus.

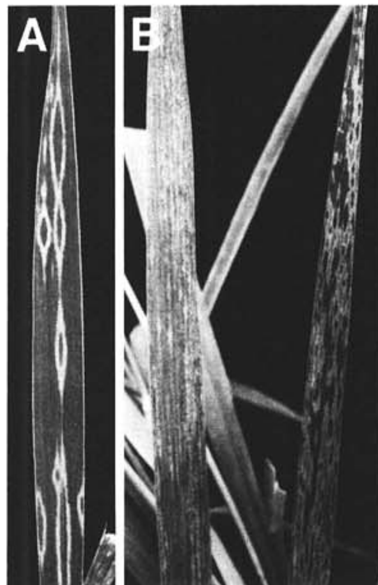

Fig. 1. (A) Characteristic eye-shaped lesions and **(B)** ensuing variegation induced by guineagrass mosaic virus in *Brachiaria brizantha.*

The virus was transmitted by *M. persicae* from infected to healthy *B. brizantha* (three of six plants infected) but not to *Sorghum bicolor* cv. Rio or sugarcane. None of the *B. brizantha* seed lots tested serologically for the presence of the virus reacted positively in DAS-ELISA. The grow-out seed test yielded 50 symptomless seedlings, all free of virus, as determined by serology.

The virus was purified in quantities exceeding 50 mg/kg of infected *B. brizantha* tissue. The average absorbance 260/280 value was 1.30, uncorrected for light scattering. Electrophoretic analyses of purified virus preparations demonstrated the occurrence of capsid protein heterogeneity consisting of four bands with approximate molecular weights of 41, 34, 32, and 30 kDa (Fig. 3A). When a purified virus preparation was intentionally maintained at room temperature (av. 25 C) for 3 days prior to SDS-PAGE, only a single band of approximately 30 kDa was observed, although further proteolytic activity was evident as well (Fig. 3B).

A clear precipitin reaction was observed in double immunodiffusion tests when the homologous antiserum was tested with tissue extracts of virus-infected *Brachiaria* spp. plants. Precipitin lines were not observed with the virus-free *Brachiaria* tissue extracts or with the guineagrass mosaic virus antisera tested. In SSEM tests, a 47.5-fold increase in the number of virus particles trapped by homologous antibodies was observed relative to the untreated control. The *Brachiaria* virus was specifically recog-

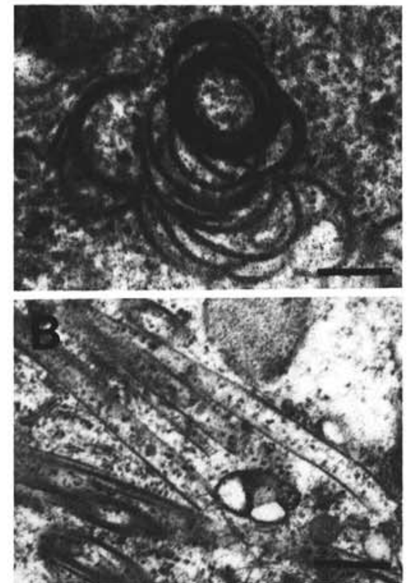

Fig. 2. Electron micrographs of a *Brachiaria brizantha* leaf cell infected by a Colombian isolate of guineagrass mosaic virus showing cytoplasmic cylindrical inclusions appearing **(A)** as "scrolls" in cross section (scale bar = 125 nm) or **(B)** as "tubes" in longitudinal section (scale bar = 400 nm).

nized by its specific γ-globulin (0.84 mg/ml) and conjugate (1 mg/ml) in DAS-ELISA tests, resulting in A_{405nm} values of 0.2–0.4, 30 min after addition of the substrate. The average A_{405nm} values of healthy *Brachiaria* tissue were 0.02. High values (A_{405nm} of 1.4–1.8 15 min after addition of substrate) were obtained in DAS-ELISA with the *Brachiaria* virus when the JTC-185 guineagrass mosaic virus antiserum was used. Under the same experimental conditions, lower but positive values were observed with the BK-215 (A_{405nm} of 0.5–0.7) and BK-203 (A_{405nm} of 0.03–0.2) antisera to guineagrass mosaic virus in the DAS-ELISA test. In PTA-ELISA, the A_{405nm} values obtained for the *Brachiaria* virus were 0.2 and 0.004 for the positive and negative controls, respectively, using homologous antiserum. Although this antiserum did not react (A_{405nm} of 0.013) with watermelon mosaic virus 2 (WMV-2) in PTA-ELISA, the four WMV-2 antisera tested readily recognized (A_{405nm} of 0.28–0.50) the *Brachiaria* virus. The commercial antipotyvirus monoclonal antibody did not react with the *Brachiaria* virus in DAS-ELISA.

Of seven desiccated *Brachiaria* spp. leaf samples collected in Brazil, five reacted positively in SSEM tests, using the antiserum prepared to the *Brachiaria* virus isolated in Colombia. Several *Brachiaria* spp. samples from Santander de Quilichao, Colombia, were also shown to be naturally infected by the virus in SSEM and ELISA tests. The *Brachiaria* virus did not react with the antisera to maize dwarf mosaic, sorghum mosaic, and sugarcane mosaic viruses used in this investigation. This virus, however, was specifically recognized by the three antisera (JTC-185, BK-203, and BK-215) to guineagrass mosaic virus, resulting in 8.75-, 20.83-, and 25.8-fold increases, respectively, in the number of virus particles trapped in SSEM tests, with respect to the untreated control.

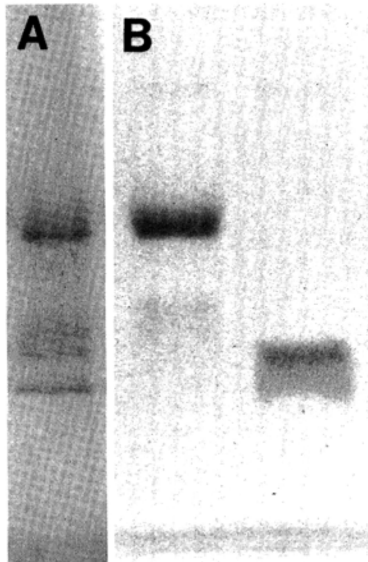

Fig. 3. SDS-PAGE of the capsid protein of a Colombian isolate of guineagrass mosaic virus showing **(A)** capsid protein heterogeneity (M_r of protein bands from top to bottom: 41, 34, 32, and 30) and **(B)** effect of limited proteolysis on the number and M_r of capsid protein species resolved (left lane = virions dissociated immediately after purification, right lane = purified virions dissociated after 72 hr of incubation at room temperature).

DISCUSSION

The particle morphology, cytopathology, mechanical and biological transmission properties, and antigenic relationships indicate that the causal agent of this previously unknown disease of *Brachiaria* spp. in Latin America is an aphid-transmitted potyvirus. The differential behavior of its capsid protein following SDS-PAGE is a phenomenon already reported for other aphid-borne potyviruses (1,16). As previously demonstrated for potato virus Y and tobacco etch virus (12), both with normal capsid protein behavior in SDS-PAGE tests, and for dasheen mosaic potyvirus (F. J. Morales, *unpublished*), which displays considerable coat protein heterogeneity (11), it was shown here for the *Brachiaria* potyvirus that a single protein species of approximately 29 kDa can be produced by limited proteolysis. The failure of the anti-potyvirus monoclonal antibody to recognize the *Brachiaria* potyvirus is not surprising, since there are other aphid-transmitted potyviruses that are not detected by this monoclonal antibody (13).

Potyviruses known to infect *Brachiaria* spp. are guineagrass mosaic virus (20), johnsongrass mosaic virus (19), maize dwarf mosaic virus (18,22), and sugarcane mosaic virus (18). Despite recent proposals for a new classification of some of these potyviruses (11), the failure of the *Brachiaria* potyvirus to infect maize, sorghum, or sugarcane and the serological tests conducted in this study indicate that the potyvirus infecting *Brachiaria* spp. in Colombia and Brazil is closely related to guineagrass mosaic virus (GGMV) but not to maize dwarf mosaic, johnsongrass mosaic, and sugarcane mosaic viruses.

The *Brachiaria* isolate of GGMV shares some characteristics with strains A and D of African GGMV. With strain A, the characteristic is to induce chlorotic eye-shaped lesions on affected leaves (20). With strain D (8), the characteristic is the production of cylindrical inclusions (scrolls) typical of subdivision I of the potyvirus group (6). In this respect, as first pointed out by Edwardson and Christie (5), the interpretation by Kukla et al (8) of the inclusions induced by GGMV-D in infected pearl millet cells as type III was incorrect. Unfortunately, the strain identification of the GGMV antisera used in this investigation was lost (J. Dubern, *personal communication*). Also, the GGMV antisera were preserved in glycerol, which probably interfered with the comparative serological double diffusion tests in agar gels containing SDS. Finally, the results of the seed-transmission tests conducted here support previous reports on the African GGMV isolates, suggesting that this virus is not seedborne (8,9,20). However, *Brachiaria* spp. are also vegetatively propagated (2).

In conclusion, the causal virus of the variegation observed on *Brachiaria* spp. in Colombia and Brazil is an aphid-borne potyvirus serologically related to guineagrass mosaic virus from West Africa (20). This is the first report on the occurrence of this potyvirus in South America.

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