Purification and Properties of a Virus from El Salvador that Causes Mild Mosaic in Bean Cultivars

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ABSTRACT

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A virus that has been named bean mild mosaic virus (BMMV), was isolated from bean *(Phaseolus vulgaris)* plants imported from El Salvador. The BMMV occurred in a mixture with another virus, bean curly dwarf mosaic virus, which was primarily responsible for the field symptoms consisting of plant dwarfing and leaf curling. Alone, BMMV produced only a faint mosaic in some experimentally inoculated bean cultivars and was latent in others. It also infected six of 18 other legume species. Of 42 nonleguminous species tested, only *Chenopodium quinoa* and *Gomphrena globosa* were infected. The virus was infectious in crude bean sap after being heated 10 minutes at 84 C, after incubating at room temperature for 6 weeks, and after dilution to 1×10^{-8}. Bean mild mosaic virus was not serologically related to any of 12 other viral pathogens of legumes that were tested. The virus had particles 28 nm in diameter. It was transmitted by the Mexican bean beetle, *Epilachna varivestis,* and by the spotted cucumber beetle, *Diabrotica undecimpunctata howardi.* Extracted nucleic acid (which had a nucleotide ratio of A = 25.8, C = 31.5, G = 21.7, and U = 21.0%) was infectious at 0.01 μg/ml, but it was not infectious after incubation with RNase for 5 minutes.

Additional key words: electron microscopy, insect transmission.

In 1971, several viruslike diseases were observed among bean cultivars (*Phaseolus vulgaris* L.) growing at the Central American Uniform Bean Varietal Nursery at San Andres, El Salvador. Symptoms on some of these diseased plants resembled those caused by bean common mosaic virus and bean golden mosaic virus (1). Other plants showed extreme dwarfing, mosaic, leaf rugosity, and curling (7) (Fig. 1). Tissue from plants with the latter symptoms was taken, under quarantine, to the Beltsville Research Center and the Plant Introduction Station for study. Two viruses were isolated readily from many of the samples. The virus that caused the curly dwarf mosaic symptoms is described elsewhere (7). This report describes a highly infectious beetle-transmitted virus that causes only mild mosaic or no symptoms among inoculated bean cultivars. Because this virus has not been described, its properties are reported here and we have provisionally named it bean mild mosaic virus (BMMV).

MATERIALS AND METHODS

The BMMV isolate was obtained from diseased plants of the El Salvador bean cultivar 27R. Antisera to viruses were produced in our laboratories, provided by colleagues, or obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, as explained previously (9, 10). Of the 30 antisera tested, 12 were to virus pathogens of legumes. They were: alfalfa mosaic, bean pod mottle, broad bean mottle, broad bean wilt, cowpea mosaic, cowpea chlorotic mottle, Echtes Ackerböhnenmosaik, peanut stunt, red clover mottle, southern bean mosaic, blackgram mosaic from H. Scott, and bean mosaico rugoso from Gámez (4).

Spotted cucumber beetles (*Diabrotica undecimpunctata howardi* Barber) were collected locally from squash blossoms and caged on beans. Mexican bean beetles (*Epilachna varivestis* Mulsant), leafminers (*Liriomyza munda* Frick), and greenhouse whiteflies (*Trialeurodes vaporariorum* Westwood), were obtained from laboratory colonies reared on lima beans. Sources of anion exchange resin for base ratio studies, other specialized chemicals, and equipment used in this study have been described (5, 9).

Bean mild mosaic virus was maintained in bean cultivars Topcrop, Stringless Green Refugee, and Columbia Pinto. Infectious sap from these cultivars was the source of virus for insect transmission, host-range, stability in vitro, and purification studies. Inoculated test cultivars and species either were back-indexed on Topcrop bean and *Dolichos lablab* L., or juice from the experimental species was tested with the BMMV antiserum produced during these studies.

Insect transmission.—Virus-free spotted cucumber beetles were caged on mechanically infected source bean plants for 1 day, then groups of three to five beetles were

caged for another day on three healthy plants in the primary leaf stage.

Mexican bean beetle larvae or adults were fed for 4 to 24 hours on BMMV-infected source plants, then caged on healthy seedling beans for definite periods or until they caused feeding injuries.

Transmission by leafminer, *Liriomyza munda* Frick, and greenhouse whiteflies, *Trialeurodes vaporariorum* Westwood was tested. About 100 female leafminers and 1,000 adult whiteflies were fed in separate experiments for 24 hours on infected plants and then caged on healthy plants for 24 hours.

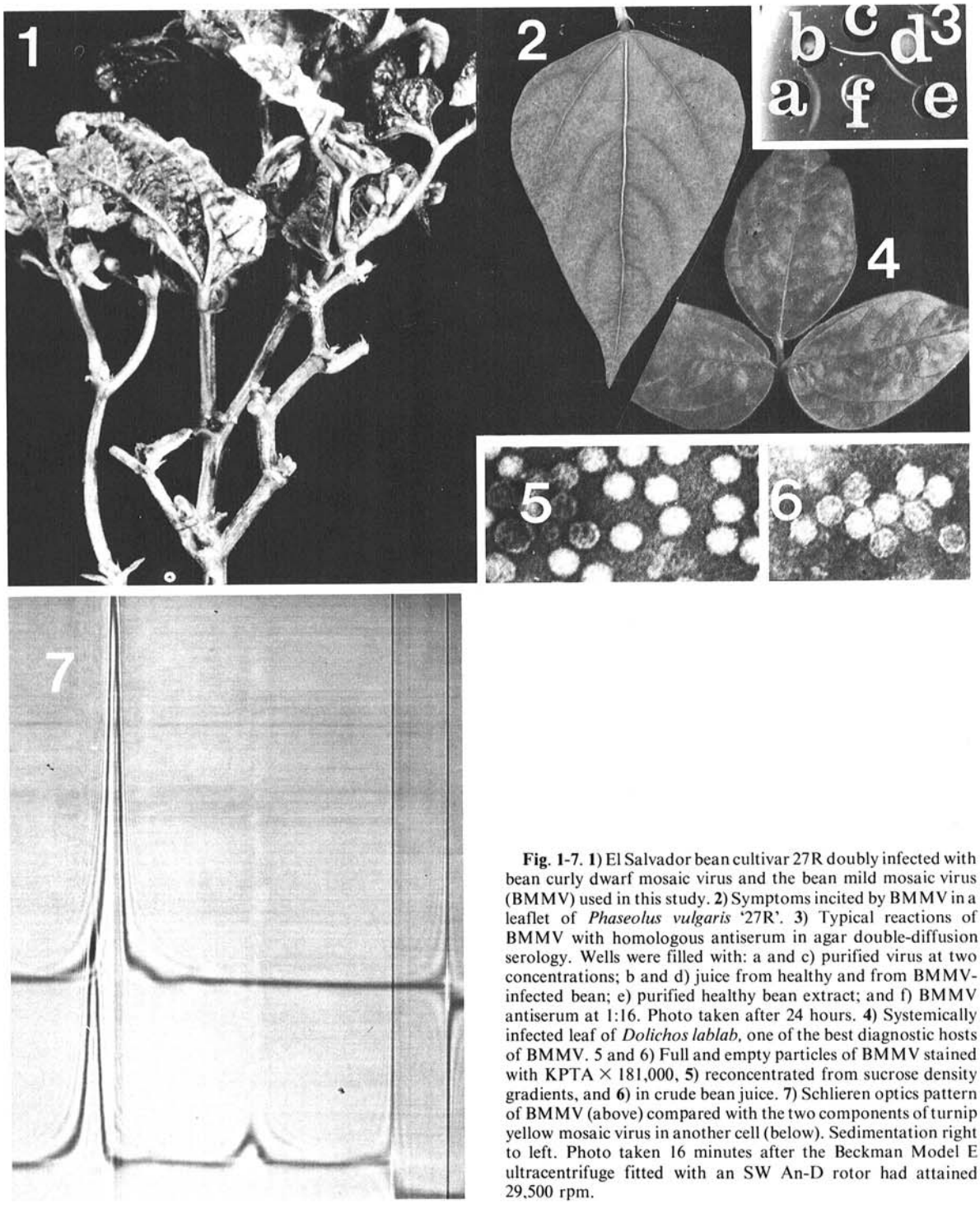

Fig. 1-7. **1)** El Salvador bean cultivar 27R doubly infected with bean curly dwarf mosaic virus and the bean mild mosaic virus (BMMV) used in this study. **2)** Symptoms incited by BMMV in a leaflet of *Phaseolus vulgaris* '27R'. **3)** Typical reactions of BMMV with homologous antiserum in agar double-diffusion serology. Wells were filled with: a and c) purified virus at two concentrations; b and d) juice from healthy and from BMMV-infected bean; e) purified healthy bean extract; and f) BMMV antiserum at 1:16. Photo taken after 24 hours. **4)** Systemically infected leaf of *Dolichos lablab*, one of the best diagnostic hosts of BMMV. 5 and 6) Full and empty particles of BMMV stained with KPTA × 181,000, **5)** reconcentrated from sucrose density gradients, and **6)** in crude bean juice. **7)** Schlieren optics pattern of BMMV (above) compared with the two components of turnip yellow mosaic virus in another cell (below). Sedimentation right to left. Photo taken 16 minutes after the Beckman Model E ultracentrifuge fitted with an SW An-D rotor had attained 29,500 rpm.

Purification.—Fresh leaves of *P. vulgaris* were harvested 10 to 20 days after inoculation and blended in two to three volumes (w:v) of 0.02 M sodium citrate buffer, pH 7.5, containing 0.02 M 2-mercaptoethanol. During the blending, we added cold chloroform to the homogenate at 20 ml per 100 g tissue. After the slurry was centrifuged at 10,000 *g* for 10 minutes, virus in the clear yellow supernatant liquid was concentrated by centrifuging at 105,000 *g* for 1.5 hours, or by precipitation with 10% (v:w) PEG 6000 (polyethylene glycol, mol wt 6,000). In the latter procedure, the preparation then was centrifuged at 12,000 *g* 30-60 minutes after the PEG had dissolved at 4 C. Virus pellets were resuspended in 0.02 M citrate buffer–an amount equal to a 40-fold concentration of the extract–the pellets were combined, and after 4 to 24 hours, centrifuged at 8,000 *g* to remove plant materials.

Concentrated virus at 2-4 mg/ml was purified by layering 0.5 to 1.0 ml onto 10% to 40% linear sucrose gradients in 0.02 M neutral citrate buffer and centrifuging in a swinging bucket rotor at 100,000 *g* for 2 hours. Gradients were fractionated and monitored with an ISCO system (Instrumentation Specialties Company, Lincoln, NB 68505). Most of the sucrose was removed from the fractions by dialysis and the virus was reconcentrated by high-speed centrifugation as above.

Immunization and serology.—Antiserum to BMMV was produced by immunizing a rabbit once a week with 3-4 mg of virus in 1 ml of suspension emulsified with 1 ml of Freund's incomplete adjuvant. The rabbit received four intramuscular injections. Bleedings began with the third injection. Serological tests were performed using the gel double-diffusion method in plates with wells 5 mm apart in 0.75% Ionagar No. 2 in water containing 0.02% sodium azide.

Electron microscopy.—Naturally infected beans showing symptoms of infection by bean curly dwarf mosaic virus (BCDMV) in the field in El Salvador were sampled for electron microscopy examination. Leaves were chopped on a glass microscope slide in the presence of 2% phosphotungstic acid (PTA) adjusted to pH 7.0 with KOH. The chopped tissue was squeezed between two glass slides and a drop of extract was placed on a Formvar-coated grid. The drop was further diluted with potassium-phosphotungstic acid, dried with a filter paper, and examined.

Fractions of purified virus in 0.02 M citrate buffer were reconcentrated from density-gradient columns and stained as above. Size of the virions was determined from magnifications obtained from polystyrene latex spheres as an internal size standard and a diffraction grating replica.

Ribonucleic acid.—Ribonucleic acid was extracted from BMMV by a modification of the phenol method (2). Ten to 15 mg of purified virus was mixed with a final concentration of 0.1 M neutral sodium phosphate buffer and 5% sodium dodecylsulfate (SDS) containing a trace of bentonite. To this preparation was added an equal volume of water-saturated phenol and the mixture was vigorously shaken for 5 minutes. The balance of this procedure has been described (2, 10). Procedures used to determine the effect of RNase on extracted RNA and on intact virus, and to determine base ratio of the RNA also have been described (9). All samples were bioassayed on Topcrop bean.

Molecular weight of BMMV RNA was determined by polyacrylamide gel electrophoresis experiments (5). Ten to 25 μg of the test RNA was layered onto 2.4% gels with and without the multiple-component marker RNA of known molecular weights from cucumber mosaic virus (CMV). Molecular weight was established as a function of distance travelled through the gel compared with CMV RNA (5).

RESULTS

Symptoms and host range.—All 25 bean cultivars, including nine from Latin America that were inoculated mechanically with BMMV, became systemically infected. Reactions among cultivars ranged from symptomless infection to barely discernible mild mosaic (Fig. 2). Presence of virus was ascertained by serology (Fig. 3). Sometimes slight vein-banding or roughening of the leaf surface also was observed. Chlorotic local lesions sometimes developed on inoculated primary leaves, evidently depending upon environmental conditions. No severe malformation of leaves or stunting of plants occurred.

Six other species of legumes were susceptible to BMMV. *Phaseolus acutifolius* Gray (Red Tepary) and *Dolichos lablab* L. showed diagnostic systemic mosaic (Fig. 4), leaf deformation, and stunting; *Macroptilium lathyroides* (L.) Urb. exhibited chlorotic local lesions of inoculated primary leaves and symptomless systemic infection; *Glycine max* Merr. 'Kanrich' and 'Scott' exhibited mild leaf mottle; and *Canavalia ensiformis* DC. and *Sesbania exaltata* (Ref.) Cory became systemically infected, but were symptomless.

Twelve species of legumes were immune to BMMV. They were *Arachis hypogaea* L. 'Florunner' and 'Tifspan'; *Cicer arietinum* L.; *Crotalaria juncea* L.; *Lathyrus sativus* L.; *Lens culinaris* Medic.; *Macroptilium atropurpureus* (DC) 'Siratro'; *P. coccineus* L. 'Achievement', 'Harrison Tenderpod', and 'Kelvedon Marvel'; *P. lunatus* L. 'Fordhook 242', 'Henderson Bush', 'Dixie Butterpea', and 'Jackson Wonder'; *Pisum sativum* L. 'Alaska' and 'Perfected Wales'; *Vicia faba* L. 'Bell'; *Vigna radiata* (L.) Wilezek. 'Berken', 'Moren', and 'Thailen'; and *Vigna unguiculata* (L.) Walp. 'Blackeye #5' and 'Yardlong'.

The only nonleguminous hosts susceptible to BMMV were *Gomphrena globosa* L. and *Chenopodium quinoa* Willd. Bean mild mosaic virus did not infect *Ageratum houstonianum* Mill., *Althea* sp., *Antirrhinum majus* L., *Amaranthus caudatus* L., *Atropa belladonna* L., *Beta vulgaris* L., *Brassica rapa* L., *Calendula officinalis* L., *Capsicum annuum* L., *C. frutescens* L., *Catharanthus roseus* G. Don., *Celosia cristata* L., *Coleus blumei* Benth., *Cucurbita maxima* Duchesne, *Cucumis sativus* L., *Cynoglossum amabile* Stapf & Drum., *Dahlia excelsa* Benth., *Datura stramonium* L., *Dianthus barbatus* L., *Hibiscus cannabinus* L., *H. acetocella* Welw., *Hordeum vulgare* L., *Lobelia erinus* L., *Lycopersicon esculentum* Mill., *Malva* sp., *Momordica balsamina* L., *Nicotiana tabacum* L., *N. glutinosa* L., *Pelargonium zonale* (L.) L'Her, *Petunia hybrida* Vilm., *Physalis floridana* L., *Raphanus sativus* L., *Salpiglossis sinuata* Ruiz & Pav., *Salvia splendens* Ker., *Sesamum indicum* L., *Tagetes erecta* L., *Tetragonia expansa* Murr., *Torenia fournieri* Lind., *Zea mays* L., or *Zinnia elegans* Jacq.

Stability in vitro.—Bean mild mosaic virus in sap extracted from and bioassayed on Topcrop bean plants was infectious after diluting 1:10 with 0.025 M phosphate buffer and heating at 84 C for 10 minutes, after incubating at room temperature for 6 weeks, and after further dilution to 1×10^{-8}.

Purification and electron microscopy.—Each of the purification procedures resulted in infectious virus consisting of complete and RNA-devoid particles and averaging 28 nm in diameter (Fig. 5). Similar particles were observed in leaf-dip preparations from the original field plants (Fig. 6).

Following rate-zonal density gradient centrifugation, only a single opalescent band was visible; and, when gradients were fractionated, a single ultraviolet-absorbing region was observed. The specific extinction coefficient of BMMV was not determined. However, if one assumes that the RNA content was between 20% and 35%, and corresponding specific extinction coefficients of $E^{0.1\%}_{260\ nm} = 5$ to 8 are used, yields were 300 to 500 mg/kg of tissue, respectively. With respect to virus yield, bentonite was a better clarifying agent than chloroform, but later concentration by PEG 6000 or by ultracentrifugation were equally effective. The ultraviolet-absorption spectrum was typical of that for nucleoprotein. The average $A_{260/240}$ ratio was 1.23 and the $A_{260/280}$ ratio was 1.52. Sedimentation coefficient of BMMV was $S_{20,w} = 129$ (Fig. 7).

Serology and relationships to other viruses.—Antiserum to BMMV had a titer of 1:64 in the first bleeding and increased to 1:2,048 with the third weekly bleeding. The antiserum had a titer to healthy plant protein of less than 1:2. Gel-diffusion serology was a reliable technique for detecting BMMV in crude juice of infected plants, whether or not symptoms were present. Virus in a few drops of extracted crude juice reacted with the homologous antiserum diluted 1:8 to 1:32 and produced a single curved band in gel-diffusion tests within 10 hours. When the ring-interface technique was used in 1-ml tubes, distinct reactions were observed within 10 to 20 minutes.

Purified BMMV, at several concentrations up to 3 mg/ml, did not react with antisera to other spherical viruses of legumes, including those causing bean pod mottle, southern bean mosaic, cowpea chlorotic mottle, bean mosaico rugoso (4), red clover mottle, echtes Ackerböhnemosaik, cowpea mosaic, nor with antisera to other viruses in the comovirus group including radish mosaic and squash mosaic viruses.

Insect transmission.—The Mexican bean beetle transmitted BMMV to 14 of 24 Topcrop bean plants when the beetles were given 19-hour accession feeding and 21-hour inoculation feeding periods with an average of 0.7 adults per plant. In a second test, with a 96-hour accession feeding, serial transfers of 0- to 16-, 16- to 24-, and 24- to 40-hour inoculation feedings, with one adult beetle per plant, BMMV infection occurred in seven of 15, four of 15, and two of 15 bean plants, respectively.

Spotted cucumber beetles transmitted BMMV to four of 24 Topcrop bean plants in tests with a 19-hour accession feeding and a 21-hour inoculation feeding with an average of 0.7 adult beetles per plant. Transmission was confirmed by serological tests. There was no evidence of transmission by leafminers or whiteflies.

Ribonucleic acid.—The RNA of BMMV produced a typical ultraviolet absorption spectrum with an $A_{260/230}$ ratio of 2.25. Percent RNA in BMMV was not determined; however, if intact virus particles contained 35% RNA, as do other beetle-transmitted comoviruses, then yields of RNA averaged about 50% of the RNA present in the original samples. When SDS was not used during extraction, RNA yields were extremely low.

RNA extracted from BMMV infected Topcrop bean plants at concentrations of 0.01 μg/ml, but not at 0.002 μg/ml. All infectivity of preparations containing 0.7 μg/ml was destroyed by 1 μg/ml of pancreatic RNase within 5 minutes as measured by bioassay on Topcrop bean plants.

Average ratio of bases from five replicates was: A = 25.8%, C = 31.5%, G = 21.7%, and U = 21.0%. Molecular weight of BMMV RNA, as determined by polyacrylamide gel electrophoresis, was 1.27×10^6.

DISCUSSION

Of the well-described viruses, bean mild mosaic virus resembles bean pod mottle virus in its host range and the symptoms it incites in many bean cultivars. However, based on number of viral components, serological data, and several other properties, we know that the two viruses are not related.

Bean mild mosaic virus appears to be none of the viruses described by Zaumeyer and Thomas (11). Furthermore, a report by H. Scott and J. Fulton given at Cali, Colombia, in December 1975 (Fulton, *personal communication*) indicated that BMMV could not be placed into any of five serogroups of the comoviruses. The relationship of BMMV to the BPMV-related virus from *Desmodium paniculatum* is unknown (6).

We do not know how widespread BMMV is in Central America. In field samples from El Salvador collected in 1971 and 1975, BMMV occurred in combination with other viruses, such as BCDMV (7), cowpea mosaic virus, or bean mosaico rugoso virus (4). However, since BMMV was transmitted readily by beetles, it may be widespread in Central American bean fields, but overlooked because of the mild symptoms produced.

Although beetle vectors have not been investigated in El Salvador, the banded cucumber beetle, *Diabrotica balteata* Le Conte, and a flea beetle, *Cerotoma raficornis* (Oliver), are prime suspects because of their general distribution and their known role as virus vectors (3, 8). Of the beetles used in our tests, only the Mexican bean beetle is present in El Salvador. It is present in low populations in localized areas and may be responsible for some of the virus dissemination.

In greenhouse tests, BMMV alone had little effect on the growth of bean plants. However, when combined with BCDMV, symptoms were more severe than with BCDMV alone (7). Thus, the economic impact of BMMV may depend upon its combined action with other viruses.

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