Purification, serology, electron microscopy and properties of the Ampollado Strain of Bean Rugose Mosaic Virus*

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COMPENDIO

La cepa del Mosaico Rugoso del Frijol, denominada Mosaico Ampollado, se aisló de frijoles (Phaseolus vulgaris) *en San Andrés, El Salvador. Las plantas infectadas mostraron un mosaico severo con deformación de las hojas y venas, ampollado de las hojas, y formación de enaciones. El virus tuvo un punto termal de inactivación de 70 - 75°C, punto final de dilución de 10^{-4}, y longevidad* in vitro *a temperatura ambiente de 3 días. El virus se purificó de hojas infectadas de frijol por clarificación con 25% cloroformo y 10% alcohol amílico y centrifugación diferencial en tampón fosfato, 0.01M, pH 7.8. En columnas de gradientes de densidad formadas con sacarosa se observaron componentes consistentes de partículas poliédricas* ca *25 - 30 nm., que fueron infectivas.* In situ, *las partículas del virus se encontraron en las vacuolas de las células del parénquima y en el lumen de los vasos cribados y el xilema. Serológicamente el virus reaccionó con antisueros correspondientes al grupo del virus Mosaico Rugoso del Frijol, mostrando ser idéntico al Mosaico Rugoso de Costa Rica, y relacionado con las razas del Mosaico Rugoso de Guatemala y El Salvador. Asímismo reaccionó débilmente con los antisueros de los virus del Moteado de la Vaina del Frijol, y, el Mosaico del Caupi de Arkansas. Sus propiedades lo clasifican en el grupo de los comovirus como una cepa severa del Mosaico Rugoso del Frijol de Costa Rica, y, no como un nuevo virus dentro del mismo grupo.*

Introduction

SEVERAL viral diseases of beans *(Phaseolus vulgaris* L.) have been reported in El Salvador: the common mosaic (10), golden mosaic (7), rugose mosaic (8), curly dwarf mosaic (16), bean mild mosaic (18), and an unnamed new virus disease described by Granillo, Díaz, Anaya and Jiménez (10), which has been referred to as the "ampollado" mosaic (blistered mosaic).

This last disease is caused by a virus and produces severe mosaic, leaf deformation similar to 2,4 D damage, blistering of the leaves and dwarfing (10). The virus is readily transmitted mechanically and by the Chrysomelid beetles *Diabrotica balteata* Lec. and *Cerotoma ruficornis* Olivier (10). This paper describes its purification and properties which place the virus in the comovirus group as a severe strain (Ampollado strain) of Bean Rugose Mosaic Virus (BRMV-A). Preliminary reports have been published (6, 14).

Material and Methods

Virus Isolate and Properties

The virus isolate was obtained from bean plants cv '27R' which were mechanically infected at the CENTA Experiment Station in Santa Tecla, El Salvador. The

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virus was maintained on Guali bean cultivar. Infectivity assays based on local lesions were made on cv. 'ICA-Pijao'.

Properties *in vitro* were determined according to standard procedures. For the seed transmissions tests, seeds from infected 'Guali, 27R' and 'Pijao' were tested. The plants were observed for 40 days after germination.

Purification

Fresh leaves of Guali were harvested 2 - 3 weeks after inoculation. Thirty grams of infected tissue were homogenized in 0.01M EDTA buffer, pH 7.8, and filtered through cheesecloth. The filtrate was emulsified and clarified with 25% chloroform and 10% amyl alcohol (v:v) for 10 min with continuous stirring. The emulsion was broken by centrifuging at 1,100 g for 20 min. The virus in the clear brown supernatant liquid was concentrated by centrifuging at 200,000 g (^{R}av) for 45 min. The pellet was resuspended in 3 ml of the buffer and centrifuged at 10,000 g for 15 min.

The virus was further purified by density-gradient centrifugation. Density-gradient columns were made by floating layers of 4, 7, 7 and 7 ml of 100, 200, 300 and 400 mg/ml sucrose in buffer, respectively, in 1 — X 3-inch cellulose nitrate centrifuge tubes which were left standing overnight at 4°C. The viral preparation (2 ml) was floated on a column, which was centrifuged at 60,000 g (^{R}av) for 240 min in the SW 25.1 rotor of a Spinco Model L-2 ultracentrifuge at 4°C. Gradients were fractionated and scanned with an ISCO fractionator and densitometer system. The high density fractions were freed of sucrose and the virus concentrated by centrifuging at 200,000 g (^{R}av) for 60 min. The virus pellet was resuspended in buffer, centrifuged at 10,000 g for 15 min and used for infectivity, serology and electron microscopy.

Electron Microscopy

Negatively stained leaf-dip preparation was obtained by placing a piece of freshly cut infected bean leaves in a drop of neutral phosphotungstic acid.

A drop of the opalescent and infectious zones from the sucrose density gradient column was carefully transferred onto a carbon-Formvar coated grid, washed with distilled water and negatively stained either with neutral phosphotungstic acid or uranyl acetate. The leaf- dip preparation and purified virus suspension were examined in a JEM 7A, or a Zeiss EM 9 electron microscopes.

For *in situ* examination, small pieces of virus-infected bean leaves were fixed in 3% glutaraldehyde, buffered in 0.05M phosphate buffer, pH 7.2, for 1 h at 4°C, and then rinsed throughly in the same buffer at 0.2 M. Postfixation was carried out in a solution of 1% Os04 in 0.15M phosphate buffer; the fixed tissues were dehydrated in acetone and embedded in Araldite. Blocks were sectioned in a Porter-Blum MT-1 ultramicrotome equipped with an IV-IC diamond knife. Sections were stained with uranyl acetate and lead citrate, and examined in a Zeiss EM 9 electron microscope.

Immunization and Serology

Virus antiserum was prepared by immunizing two rabbits once a week with 4 - 5 mg of virus in 1 ml of suspension emulsified with 1 ml of Freund's incomplete adjuvant. Each rabbit was injected three times intramuscularly with an endovenous injection the second week of immunization. The rabbits were bled five weeks after the first injection. Serological tests were performed using the gel double-diffusion method in plates with wells 5 mm apart in 1% Special agar (Noble) containing 0.6 per cent sodium azide. Titration of the antiserum was done by the microprecipitin and gel double-diffusion tests.

The virus was tested with antisera to 14 legume viruses or strains. They were: Antisera to the bean rugose mosaic virus (BRMV-CR); bean rugose mosaic virus (BRMV-El Sal.); bean rugose mosaic virus (BRMV - Guat.); and bean golden mosaic virus (BGMV); and the following which were provided by J. Fulton and H. Scott, quail pea mosaic virus (QPMV); bean rugose mosaic virus (BRMV-Ark. antiserum); black gram virus (BGV); cowpea mosaic virus - yellow strain or mild strain - Sb. (CPMV - Sb.); cowpea mosaic virus (CPMV Ark.); cowpea TMV strain (TMV-CP); southern bean mosaic virus (SBMV); southern bean mosaic cowpea (SBMV) - CU); bean pod mottle virus (BPMV); and bean yellow stipple virus (BYSV).

Results

Stability in vitro

The virus in sap extracted from 'Guali' and bioassayed in 'Pijao' had a thermal-inactivation point of 70° - 75°C after heating for 10 min, a dilution-end point of 10^{-4}, and a longevity of 3 days at room temperature (20-22°C).

Seed Transmission

Only healthy plants were obtained out of 450 seeds harvested from diseased plants of the cultivars 'Guali', '27-R', and 'Pijao'.

Purification

Two opalescent bands at 24.5 and 28.8 mm below the meniscus were seen in rate-zonal density-gradient tubes but when the bands were fractionated and scanned at 254 nm with ultraviolet light, two distinct viral components were resolved (Fig. 1). Both components

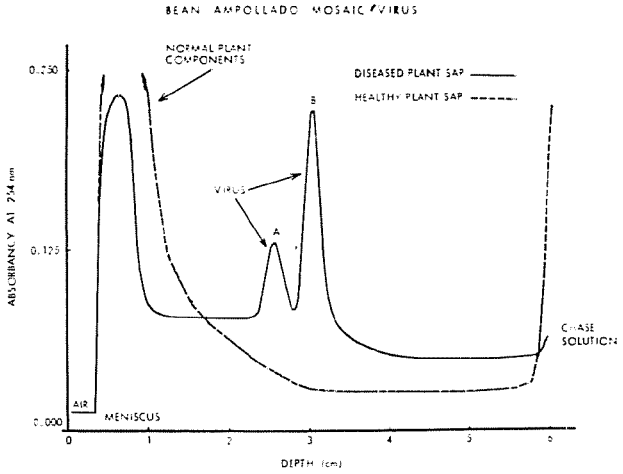

Fig. 1.—*Scanning pattern of healthy and BRMV-A infected leaf extracts after chloroform -amyl alcohol clarification, followed by differential and density -gradient centrifugation. Occasionally 3 components were detected.*

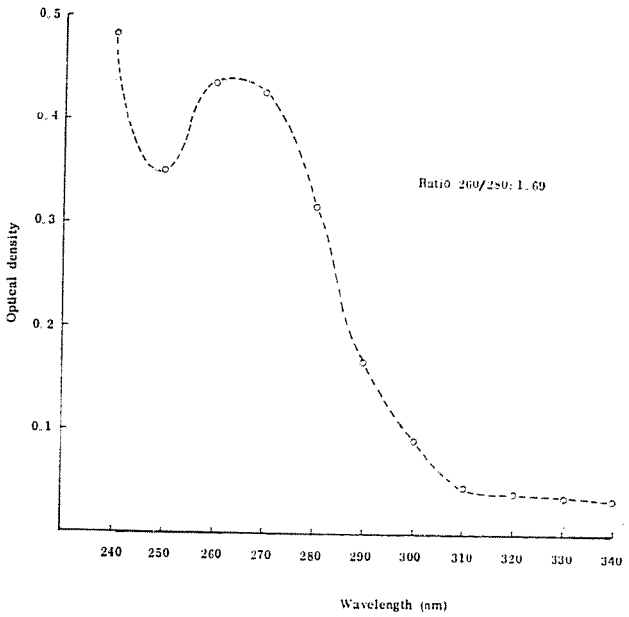

Fig. 2.—*Ultraviolet absorption spectrum of purified Bean Rugose Mosaic Virus, "Ampollado strain". (Bottom component).*

were infectious, the bottom component being more infectious than the top one. Both components gave an ultraviolet-absorption spectrum typical of nucleoproteins (Fig. 2). The average A 280/260 ratio was 0.59 ± 0.1, for the bottom component. This value suggested a content of 20 - 22 per cent RNA. (17). These components were not present in gradients with healthy plant preparations.

Electron Microscopy

Isometric particles *ca.* 30 nm in diameter could consistently be detected among the cell debris in leaf-dip preparations (Fig. 3, upper insert). In purified preparations, the lighter component was composed mostly by apparently empty particles, in which the stain penetrated the core, resulting in particles with a dense center (Fig. 3, lower insert). The heavier fraction, on the contrary, was formed exclusively by unpenetrated particles (Fig. 3, 4). Both types of particles measured 25-30 nm in diameter. The presence of only a few extraneous materials indicated the degree of the purity of the preparations. When a large number of particles were grouped together, they tended to form regular hexagonal arrays (Fig. 4).

In situ observations showed no remarkable alterations of the affected tissues at low magnifications. Cells were usually somewhat shrivelled, but their components apparently had a normal shape and distribution (Fig. 5). Vacuoles usually contained dense specks, while the cytoplasm showed some highly vesiculated areas. When the cells were examined at higher magnifications, the dense vacuolar material was composed of aggregates of dense spheroidal particles, *ca.* 25 nm in diameter (Fig. 6, 7). In some instances a dense amorphous material appeared interspersed with these particles (Fig. 7). These particles were difficult to identify clearly in the cytoplasm, since they are similar in size to the ribosomes, although having a smoother profile. However, in the cytoplasmic areas rich in vesicles, they were seen to form small aggregates, often surrounded by a membrane (Fig. 8). These vesicles ranged in diameter from 0.1 to 0.5 nm, usually without visible content, but occasionally containing a thin thread mesh (Fig. 9). Another structure occasionally found associated with BRMV-A infection was a loose bundle of fibrous material about 20 nm thick in the cytoplasm, usually near the aggregate of the vesicles (Fig. 9). The isometric particles were also occasionally seen in both the sieve tubes and xylem vessels (Figs. 10 and 11). In the sieve tube they were found within membrane-bound cavities, while in the tracheid these particles appeared scattered in the vessel lumen.

Serology and Relationship to other Viruses

Antiserum to the virus had a titer of 1:256. Antiserum of the virus reacted strongly with bean rugose mosaic virus of Costa Rica, and slightly with BRMV

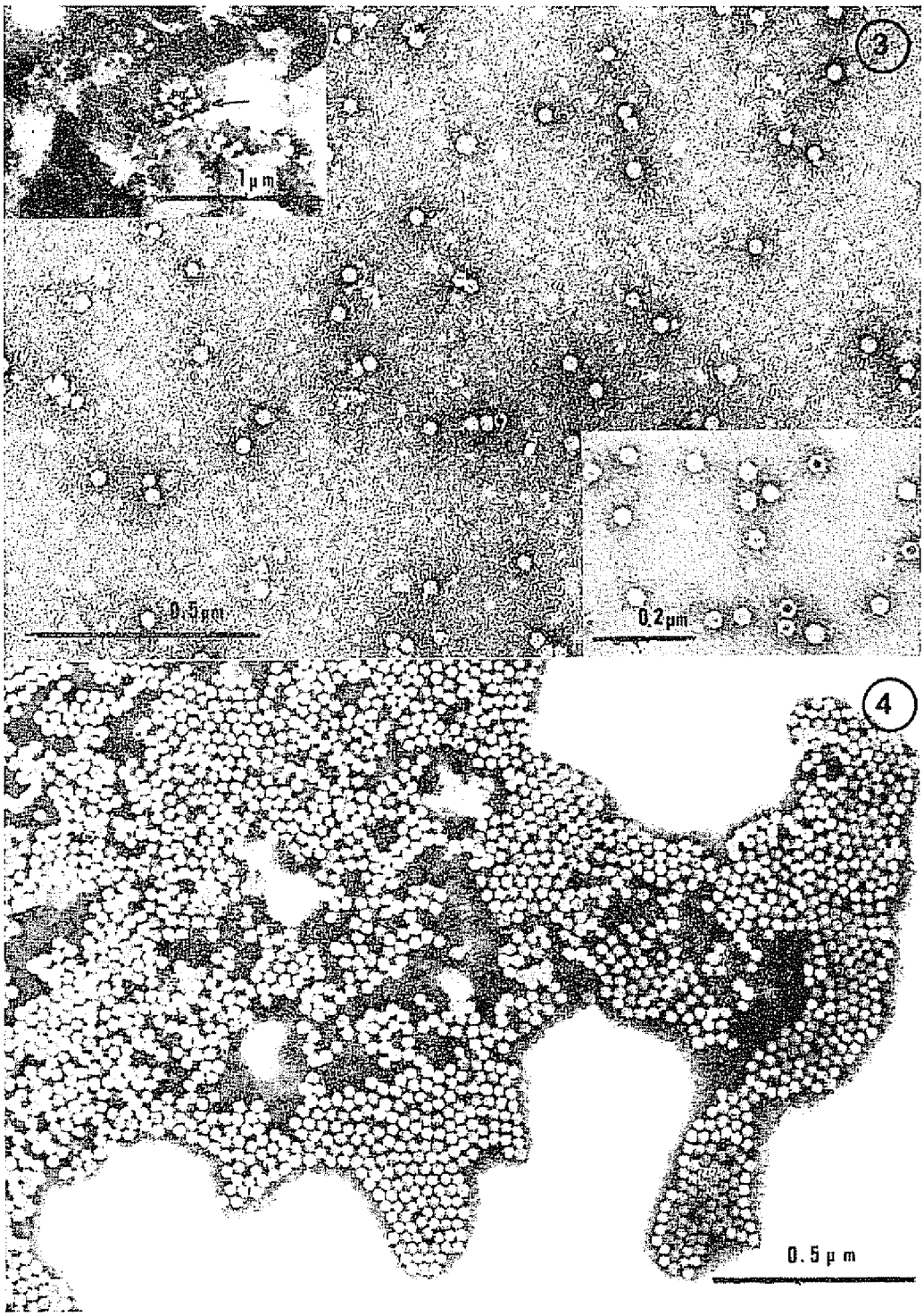

Fig. 3.—*Isometric particles in a highly purified negatively stained preparation of bean rugose mosaic virus ampollado strain. Lower insert exhibits apparently empty capsids, from a lighter, non-infectious zone, after sucrose density-gradient centrifugation. Upper insert shows some isometric particles in a leaf dip preparation.*

Fig. 4.—*Similar to Fig. 3, but showing a more concentrated preparation, in which particles tend to arrange in a hexagonal array upon drying.*

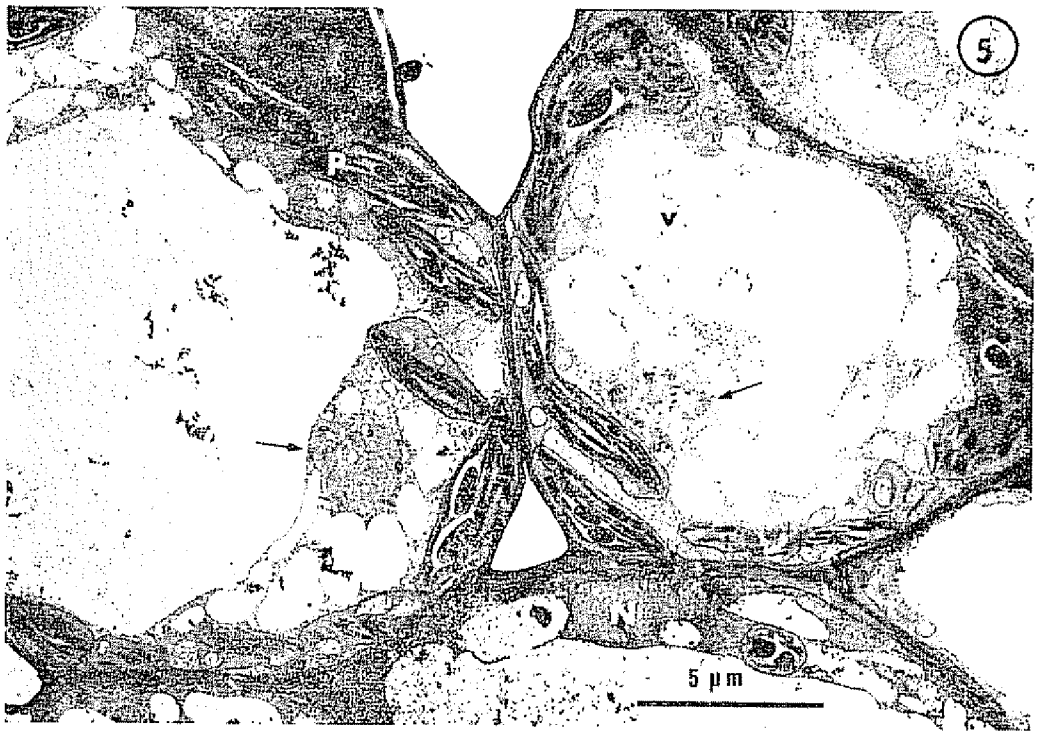

Fig. 5.—*Low magnification view of a spongy parenchyma of bean leaf, infected with BRMV-A. Note that the cells have a normal appearance, except for some cytoplasmic areas rich in vesicles (arrows). Dense specks are noticeable in the vacuole.* V- *virus particle;* N- *nucleus;* P- *chloroplast*

of Guatemala and El Salvador. It reacted weakly with bean pod mottle virus and cowpea mosaic virus Arkansas. There was no reaction to normal serum or plant healthy sap.

Discussion

Bean ampollado mosaic virus was recorded in bean fields in the San Andrés Experiment Station in El Salvador in 1973 (10). The virus is readily transmitted mechanically and by beetles (10). Infected plants resemble the 2,4 D damage syndrome and produce no yield.

The sap properties of the BRMV-A were close to those of BRMV. Symptoms in *P. vulgaris* resemble those of BRMV, A-1 strain (3); however, in some cultivars the reaction was different. A strong serological reaction was obtained with BRMV of Costa Rica, and less with BRMV of El Salvador and Guatemala. It reacted weakly with bean pod mottle virus and cowpea mosaic virus Arkansas strain. These latter reactions were similar to those reported for the BRMV Costa Rica (8).

The polyhedral particles present in the highly infectious purified preparations must be BRMV-A virions which are similar in size and shape to those of BRMV (8). The empty particles forming the non-infectious lighter zone in the sucrose density - gradient columns are probably virus particles devoid of nucleic acid. In some instances three components were resolved, one of them in very low concentration.

The particle morphology agrees with the symptomatology, transmission tests, stability *in vitro* and serological works of the bean rugose virus group which would place the studied isolate in the same virus group (3, 4, 5, 8).

Histological examination of infected leaf tissues in the electron microscope detected the presumed BRMV-A particles *in situ*. The isometric particles seen within infected cells are regarded as virions due their constant size and shape, and because their size is similar to the particles found in purified preparations. Virus particles often appeared within the vacuoles and were occasionally associated with vesicles in the cytoplasm, but their similarity in morphology with the ribosomes makes a clear cut identification difficult. The vesicles are probably involved in virus synthesis and/or assembly. Another feature of the BRMV-A infected cells was the occasional appearance of a bundleof loose fibers whose nature has not been determined yet but is similar to that reported in cells infected with cowpea mosaic virus (1,2) and strain A2 of the bean rugose mosaic virus (15).

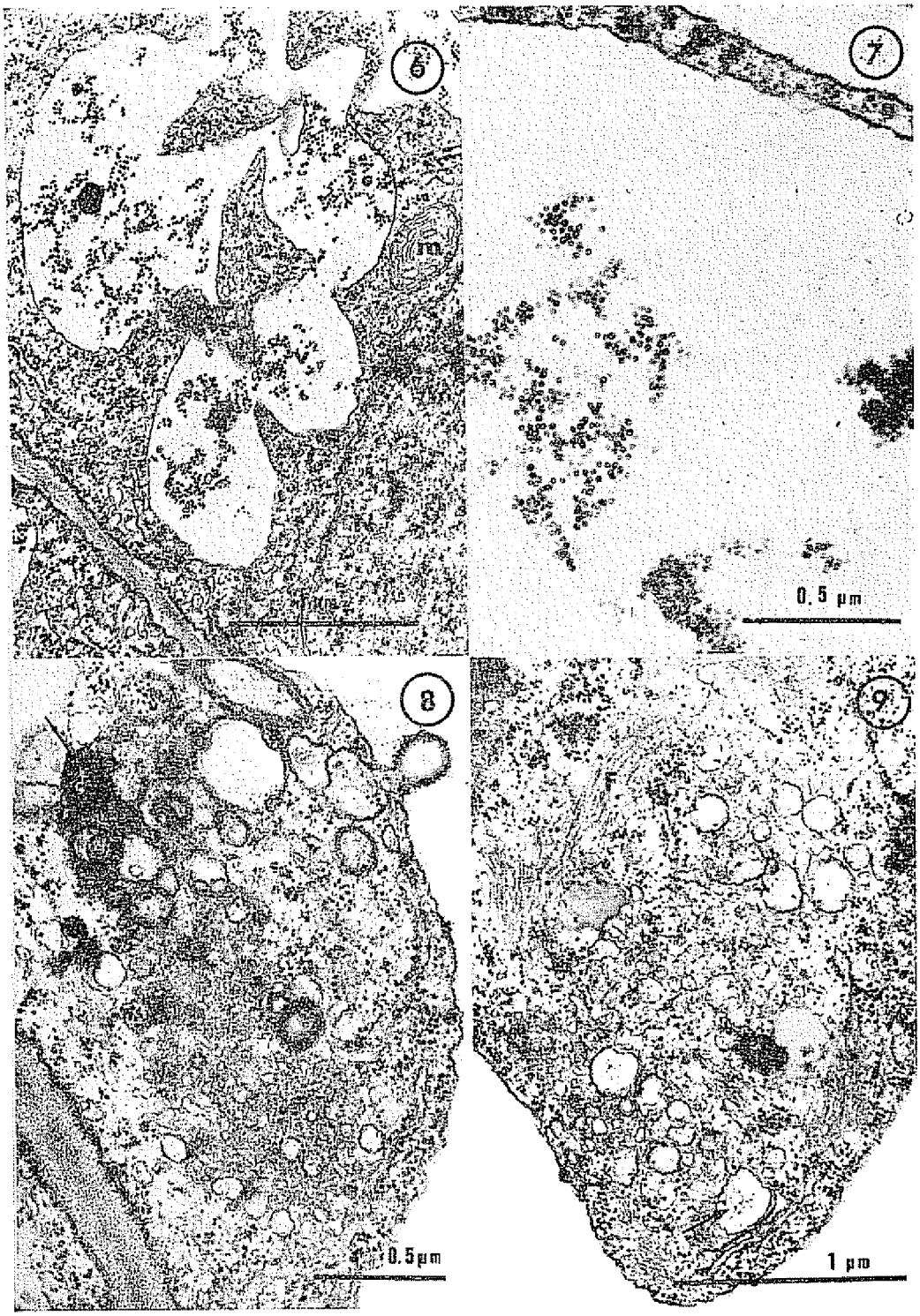

Fig 6 7 —*At higher magnifications the dense specks in the vacuole appear to be made up of isometric particles (r), probably representing BRMV-A - virions* in situ *Sometimes the particles appear interspersed with a dense amorphous material (Fig 7)* N - *nucleus* m - *mitochondrion*

Fig 8 —*Detail of the cytoplasmic area in vesicles. Note some virus like particles within membrane-bounded cavities (arrow).*

Fig 9 —*Another cytoplasmic area rich in vesicles, some of which contain a threadlike material (arrow). Loose bundles of a fibrous material* (F) *appear near the vesicles*

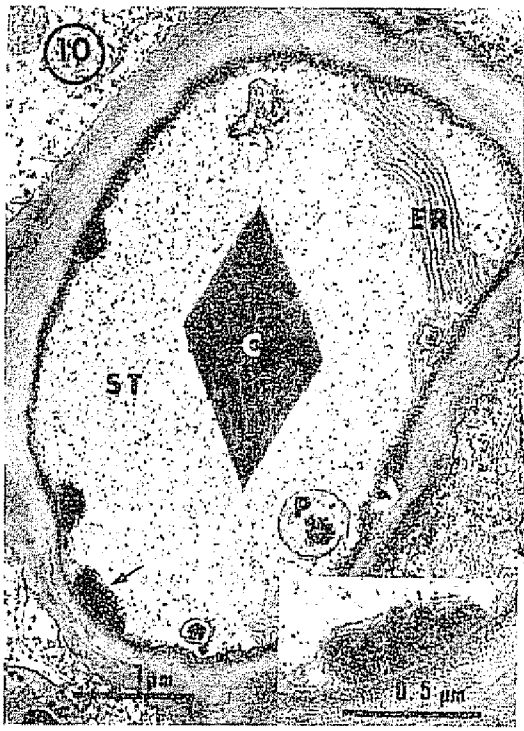

Fig. 10.—*Virus-like particles in the sieve tube (ST) within membrane bounded cavities (arrow). Insert is a detail of such a cavity. ER-endoplasmic reticulum; P- chloroplast; C - crystalline inclusion*

Fig. 11.—*Virus-like particles (v) in the lumen of a xylem vessel (X)*

The high concentration of the presumed virus particles in the infected tissue is also confirmed by the relatively easy detection of these isometric particles in leaf dip preparations, which can thus be used for diagnostic purposes

Although cytologically BRMV-A infection produces only a few of the cytopathic effects induced by the BPMV (12) to which BRMV-A is serologically related, this is not particularly significant since strains of the same virus sometimes produce different intracellular behavior (15, 19)

Physical properties, size and shape of the particle, vector, multiple component nature, and serological and relationship place BRMV-A in the comovirus group (9, 11). Furthermore, it closely resembles bean rugose mosaic virus of Costa Rica (8) Thermal inactivation point, longevity *in vitro*, and size of the virus particle distinguish it from bean curly dwarf mosaic virus (16), bean pod mottle virus (20), and its multiple component nature from bean mild mosaic virus (18), bean line-pattern mosaic virus (13), bean yellow stipple virus (7) and bean southern mosaic virus (4) Serologically it is related to the Salvadorean and Guatemalan BRMV, and to a lesser extent to BPMV and CPMV - Arkansas. All these consideration lead us to the conclusion that ampollado disease of beans is not due to a new virus but to the severe Costa Rican strain of bean rugose mosaic virus.

Summary

Bean Rugose Mosaic Virus "Ampollado Strain", BRMV-A, had a thermal inactivation point of 70 - 75°C, a dilution end point of 10^{-4}, and survived for 3 days at room temperature It was apparently not seed transmitted. It was purified by clarification with 25 per cent chloroform and 10 per cent amyl alcohol, differential centrifugation, and rate density-gradient centrifugation Two components were always isolated, which were infectious Isometric particles *ca.* 25-30 nm were seen in leaf-dip and purified preparations as well as in the cytoplasm where they form small aggregates often surrounded by a membrane The isometric particles were also observed in both the sieve tubes and xylem vessles. Serological tests showed a close relationship to BRMV, BPMV, and CPMV-Arkansas All the properties studied indicated that it is not a new virus but a severe strain of the bean rugose mosaic virus - Costa Rica strain

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