# Papaya Ringspot Virus and Cucumber Mosaic Virus Associated with a Severe Mosaic in Melon[1]

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## ABSTRACT

**Severe mosaic disease was observed in several melon fields in Costa Rica during the period 1987-1991. Two viruses, papaya ringspot virus (PRSV) and cucumber mosaic virus (CMV) were associated in single and mixed infections with the mosaic disease. These viruses were partially characterized on the basis of the type of cytoplasmic inclusions observed by light microscopy, the size and morphology of the virus particles by electron microscopy, and the serological relationships with members of the potyviruses and cucumoviruses groups using immunodiffusion and immunoelectrophoretic transfer of proteins. This is the first report of PRSV and CMV occurring on cucurbits in Costa Rica.**


**Key words: Identification, partial characterization, cucurbit viruses, potyvirus, cucumovirus.**


## COMPENDIO

**Los virus mancha anular de la papaya o "papaya ringspot virus" (PRSV) y mosaico del pepino o "cucumber mosaic virus" (CMV) fueron asociados con un mosaico severo en el caso de infecciones simples y mixtas en plantas de melón en Costa Rica, durante el período 1987-1991. Estos virus fueron identificados y parcialmente caracterizados con base en los tipos de inclusiones citoplasmáticas, observadas por microscopía de luz, en la morfología y tamaño de las partículas virales, por microscopía electrónica, y en sus relaciones serológicas con miembros de los grupos potyvirus y cucumovirus mediante inmunodifusión y transferencia inmunoelectroforética de proteínas. Esta es la primera vez que se informa sobre la presencia en cucurbitáceas del virus de la mancha anular de la papaya y del virus del mosaico del pepino en plantas de melón en Costa Rica.**


**Palabras claves: Identificación, caracterización parcial, virus en cucurbitáceas, potyvirus, cucumovirus.**

## INTRODUCTION

In the last five years, within a new program of agricultural diversification, melon has become an important economical export crop in Costa Rica. To provide a continuous supply for the export market, which is open only between November and April (dry season), growers plant melons successively at one- and two-week intervals during this season. This situation favors the spread of virus by aphid vectors. A severe mosaic was observed in several melon fields in Costa Rica during the period 1987-1991. The incidence of mosaic symptoms was 1.5% - 73.5%, causing severe losses (Rivera *et al*., unpublished data).

The watermelon mosaic virus-I (WMV-I), reclassified recently as papaya ringspot virus (PRSV) (21), and cucumber mosaic virus (9) are known to infect cucurbit crops all over the world causing severe losses in crop yield (5, 6, 7, 8, 13, 16, 18, 19). Both viruses are transmitted mechanically and by many species of aphids in a non-persistent manner.

PRSV is a member of the potyvirus group, with flexuous, filamentous particles about 780 nm long. This virus induces cylindrical (pinwheels) inclusions in the cytoplasm of host cells (2). The viral particles contain a single-stranded RNA of positive polarity and a single type of capside protein that has an apparent molecular weight of 36 K (21).

Cucumber mosaic virus (CMV) is the type member of the cucumovirus group (9). The viral particles are isometrical with a diameter of about 29 nm, built from identical protein sub-units of a molecular weight of

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about 26 K (11). The genome of CMV is composed of three single-stranded RNA molecules and subgenomic encapsidated molecule (15). This virus induces angular crystalline inclusions in the cytoplasm of host cells (2).

This study was carried out to identify and partially characterize the viruses associated with the severe mosaic disease observed in the field by different analyses: cytoplasmic inclusions by light microscopy; particle morphology and size by electron microscopy; serological relationships by immunodiffusion; and immunoelectrophoretic transfer of proteins.

## MATERIALS AND METHODS

Naturally-infected melon leaves with severe mosaic disease were collected in commercial fields during the growing seasons in Costa Rica. Virus identification by light microscopy of viral inclusions, electron microscopy of partially purified virus particles and immunodiffusion tests were carried out on field-collected samples.

To maintain the virus isolates in the greenhouse, mechanical inoculation onto carborundum-dusted zucchini (*Cucurbita pepo* L.) was undertaken using crude extracts of melon field plants with single viral infections. The inoculum was prepared in a 0.01 M sodium phosphate buffer, pH 7.2. These greenhouse isolates were used for purification and immunoelectrophoretic transfer of viral proteins.

### Light microscopy of viral inclusions

Epidermal stripes and thick tissue pieces of symptomatic field melon leaves and greenhouse healthy melon leaves were stained with Azure A and Calcomine Orange-Luxol Brilliant Green BL (OG-stain) techniques and mounted in a drop of Euparal (Carolina Biological Supply) on a glass slide, as described previously (3). The preparations were examined with a Zeiss Standard 19 light microscope. Light micrographs of the observed inclusions were carried out with a Zeiss M 35 camara.

### Electron microscopy of viral particles

Partially purified virus preparations from field-infected melon leaves were negatively stained with 1% uranyl acetate, as described previously (4), and examined under a Hitachi 12 A electron microscope. The average diameter of isometric particles and the length distribution of filamentous particles were determined by measuring directly from electron micrograph positives.

### Immunodiffusion

Diseased melon plants were tested for the presence of CMV and squash mosaic virus (SqMV) using the immunodiffusion medium of 0.8% agarose in 0.1 M borate-0.05 M ethylenediaminetetraacetic (EDTA) buffer, pH 9, containing 0.5% Triton X-100 and 0.01 M sodium thioglycolate as previous described (1). Double-diffusion tests were also conducted in sodium dodecyl sulfate (SDS), agar gel (0.8% Ionagar) containing 0.5% SDS as reported by Purcifull and Batchelor (20) for testing the presence of WMV-1, watermelon mosaic virus-2 (WMV-2), and zucchini yellow mosaic virus (ZYMV). Rabbit policlonal antisera against the four viruses were used.

### Purification of PRSV and CMV

Sixteen days after inoculation with PRSV, greenhouse zucchini leaves were harvested and purified according to the method of Leiser and Richter (14). Tissue was first frozen and then homogenized in one volume of a solution containing of 0.5 M sodium citrate, 0.005 M EDTA, 0.015 M Na-diethyldithiocarbamate (DIECA), pH 7.4. After centrifugation at 3800 *g* for 15 min, 3% vol/vol Triton X-100 was added to the aqueous phase and stirred for 30 min at 4°C. The virus was recovered by ultracentrifugation at 81 400 *g* for two hours. The pellet was suspended in 0.010 M Citrate buffer, pH 7.4, containing 1 M urea, 0.1% vol/vol mercaptoethanol and left overnight at 4°C. After clarification at 16 800 *g* the supernatant was subjected to one cycle of centrifugation in a sucrose cushion of 30% vol/vol in 0.05 M sodium-potassium phosphate buffer, pH 7.2, 0.01 M EDTA at 65 100 *g* for three hours. The virus in the pellet was suspended in 0.005 M borate buffer, pH 8, containing 0.03 M NaCL and 0.003 M sodium citrate, and clarified at 1900 *g* for five minutes.

CMV was purified from zucchini plants 10 days after inoculation according to the following method: infected tissue was homogenized in two volumes of 0.5 M citrate buffer pH 6.5 containing 0.005 M EDTA sodium salt, 0.5% thioglycollic acid. The filtrate was emulsified with 1/20 volume of cold chloroform, and after centrifugation at 4000 *g* for 5 min, the virus was recovered from the aqueous phase by 2 cycles of precipitation using 8% polyethylene glycol 6000 (PEG), 0.3 m NaCl. The pellet was resuspended in 0.5 M citrate buffer, pH 6.5, and subject to one cycle of centrifugation in a sucrose cushion of 30% vol/vol in

0.5 M citrate sodium buffer, pH 6.5, at 65 100 g for three hours. The virus recovered from the pellet was suspended in the citrate buffer.

### Immunoelectrophoretic transfer of proteins

Viral proteins from purified viruses and healthy plant proteins were dissociated and electrophoresed in a 15% gel SDS-PAGE with a ratio o acrylamide to bis-acrylamide of 30:1, using the Laemmli buffer system (12). The fractionated proteins were electroblotted to nitrocellulose sheets (24) by applying a constant voltage density of 2.5 v/cm$^2$ in a mini-trans-blot system (Bio-Rad Laboratories). Immunological detection was carried out according to the method of J. Hammond an R. L. Jordan (personal communication). The remaining protein binding site on the nitrocellulose membrane were blocked in buffer 0.02 M Tris, 0.15 M saline, (TBS) pH 7.5 containing 1% dry milk, 0.5% bovine serum albumin for 30 min at room temperature. The membranes were incubated separately for two hours with different primary antibodies (rabbit policlonal anti-CMV and anti-PRSV, and potyvirus-specific mouse monoclonal). Following three successive 10 min washes in TBS, the membranes were incubated with second antibodies labeled with alkaline phosphatase (goat anti-rabbit immunoglobulins for rabbit polyclonal antisera or goat anti-mouse immunoglobulins for mouse monoclonal antibodies) for two hours at room temperature.

The nitrocellulose membranes were again washed three times, 10 min each in TBS, before immersion in a substrate solution containing 14 mg nitroblue tetrazolium and 7 mg of 5-bromo-4-chloro-3-indolyl phosphate in 40 ml substrate buffer of 0.01 M Tris, 0.1 M NaCl, 5 mm $MgC_{12}$, pH 9.5.

The molecular weight of viral proteins was determined using SDS-PAGE molecular weight standards.

## RESULTS

### Symptom description

Naturally-infected melons showed a severe mosaic formed by chlorotic areas and green vein-banding (Fig. 1).

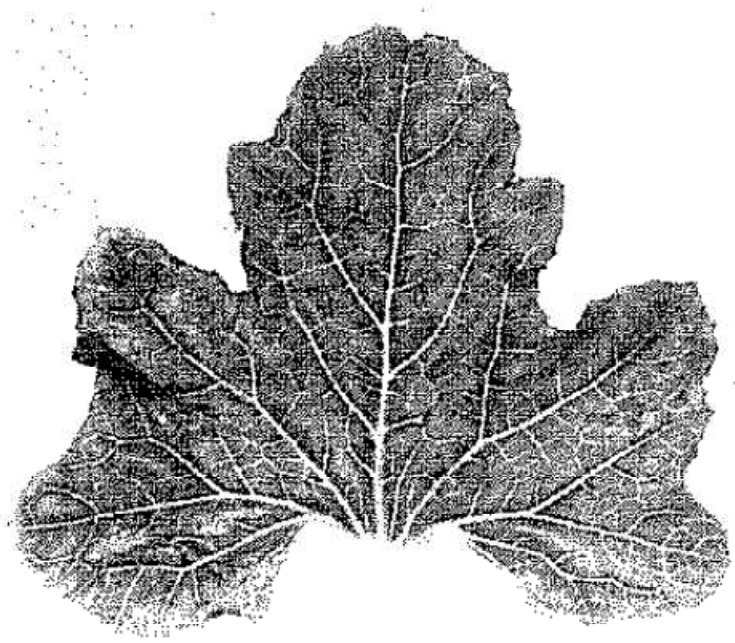

Fig. 1 Severe mosaic observed in naturally field-infected melon plants

### Light microscopy of viral inclusions

Cylindrical inclusions, similar to those reported for the potyvirus group (2) were detected in the epidermal cytoplasm when stained with OG (Fig. 2a). Aggregates of crystals in cytoplasm of epidermis and mesophyl, densely stained only with Azure A similar to those reported for cucumovirus (2) were also observed (Fig. 2b). Both types of inclusions were observed either in the same plant or separately in different plants.

### Electron microscopy of viral particles

Flexuous filamentous particles (Fig. 3a), and/or isometric particles of about 29 nm (Fig. 3b), were consistently observed in partially purified samples from naturally-infected melon plants, which are found in single and mixed infection.

From the frequency length distribution of filamentous particles, in 41 mu-particle-size groupings (Fig. 4) the mode range of 761-802 was taken as representative of the favored length for this virus with a normal length of 781.5 nm (based on 39% of particles measured).

### Immunodiffusion

No reaction was obtained between the field samples and WMV-II, XYMV and SqMV antisera when crude extracts of naturally-infected melon were used in immunodiffusion reactions. Precipitine lines were observed only when the samples were tested against PRSV and CMV antisera.

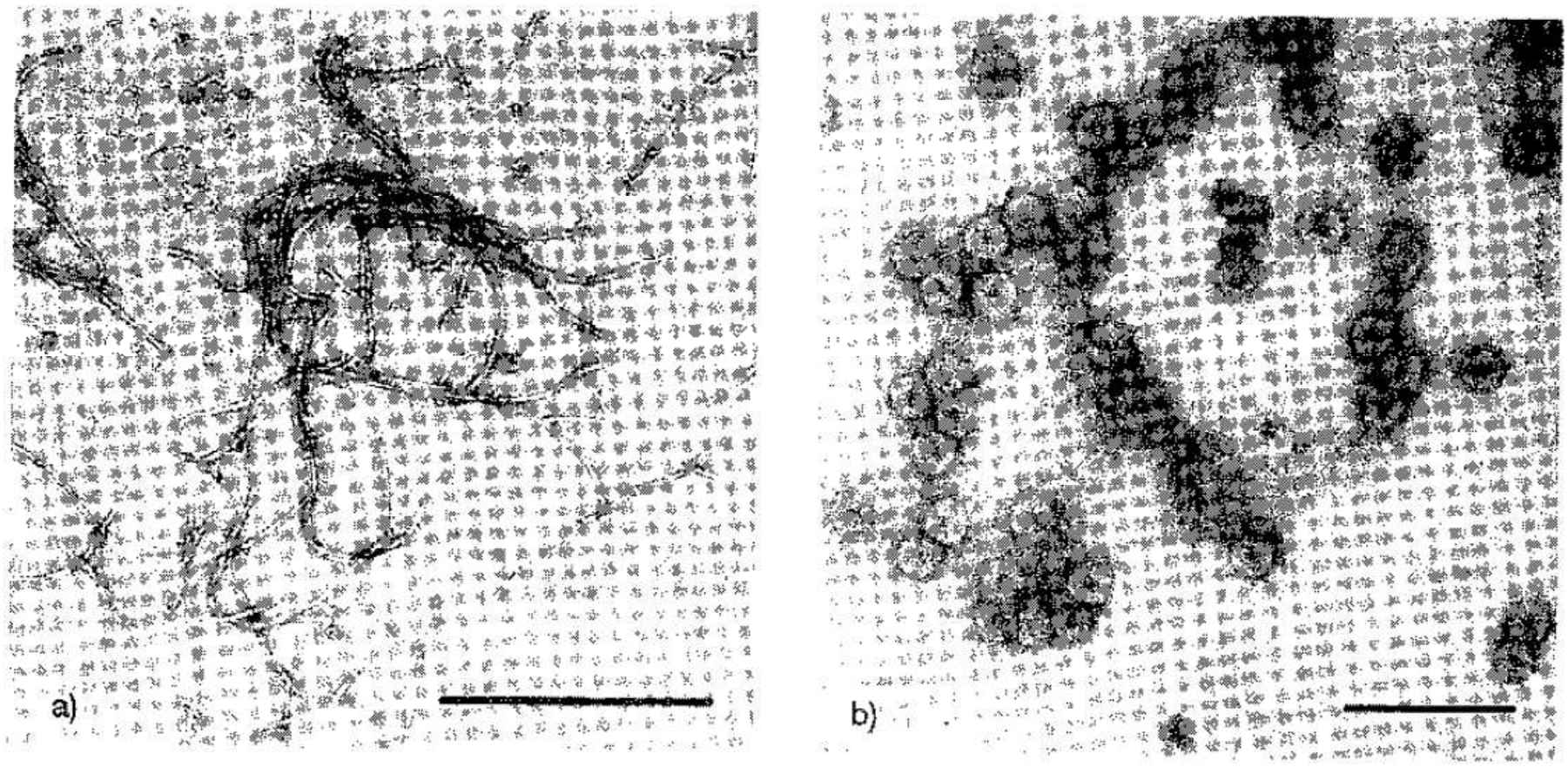


Fig. 2. Light micrographs of infected epidermal and mesophile tissues showing cytoplasmic inclusions. (a) Epidermal stripes stained with O-G, arrows point to typical masses of cylindrical inclusions; bar represents 3 um; (b) mesophile cells stained with Azure A, arrows point to densely stained aggregates of crystals; bar represents 5 um.

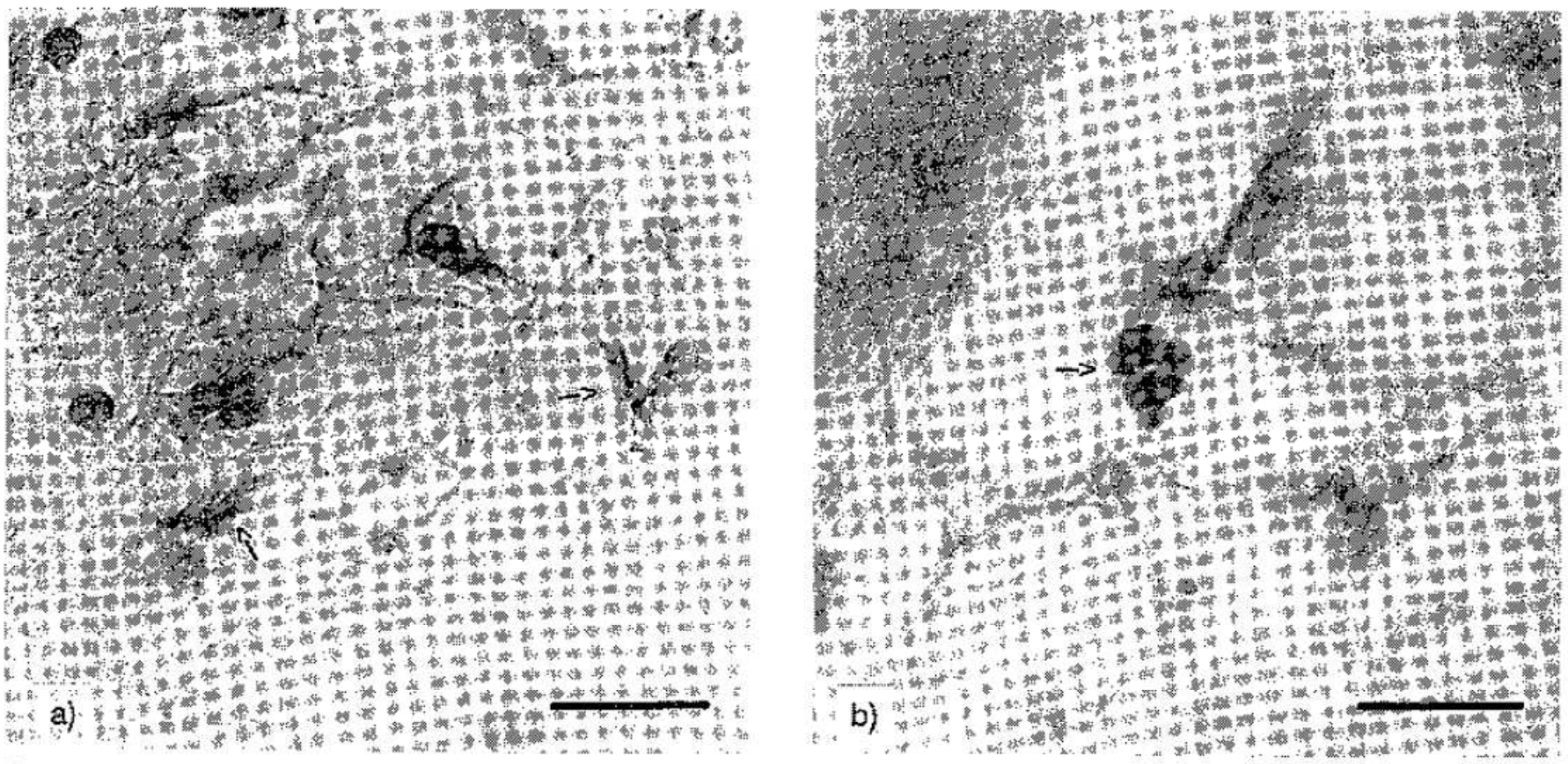


Fig. 3. Electron micrographs of viral particles observed in partially-purified preparations from naturally-infected melon. (a) Flexuous filamentous particles; bar represents 800 nm; (b) isometric particles; bar represents 100 nm.

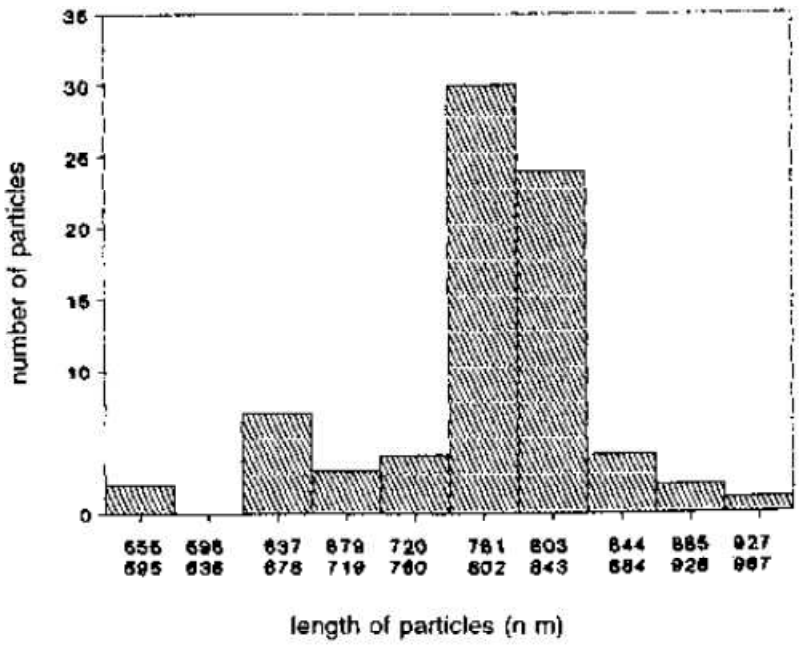


Fig. 4. Frequency distribution of length of flexuous filamentous particles.

## Immunoelectrophoretic transfer of proteins

One predominant band of 36 K and a minor protein band of 33 K were observed after the immunodetection with both the policlonal ante-WMV-1 and monoclonal PTY 1 (Fig. 5). In both cases the observed viral specific bands corresponded to the expected molecular weight of the coat protein of potyviruses (23).

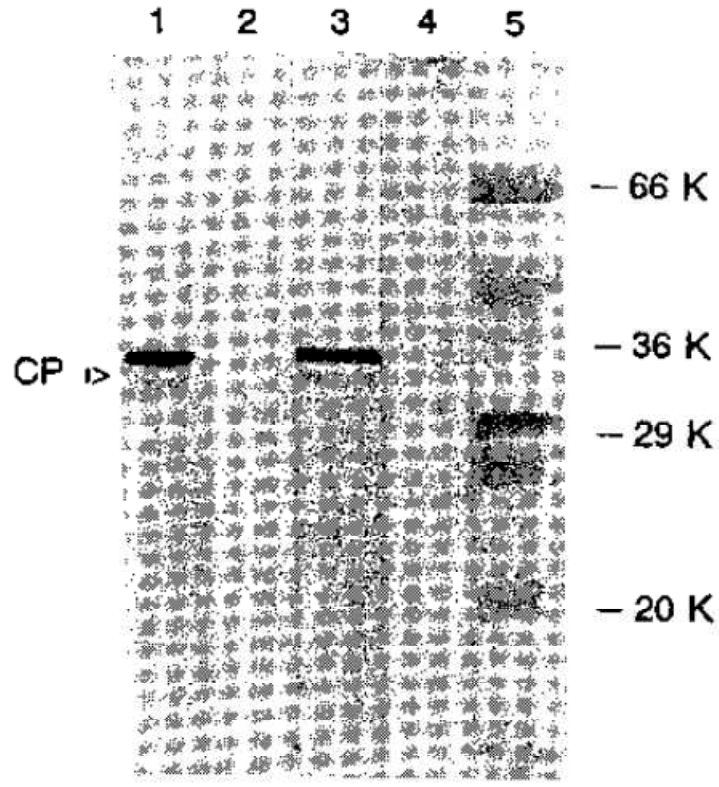


Fig. 5. Immunoelectrophoretic transfer of PRSV proteins from 15% polyacrylamide gel onto nitrocellulose sheets. Lanes 1 and 2, proteins of purified PRSW and healthy plant proteins respectively, after immunostaining with PTY-1 monoclonal antibody. Lanes 3 and 4, proteins of purified PRSV and healthy plant proteins respectively, after immunostaining with policlonal antiserum. Lane 5, protein molecular weight markers.

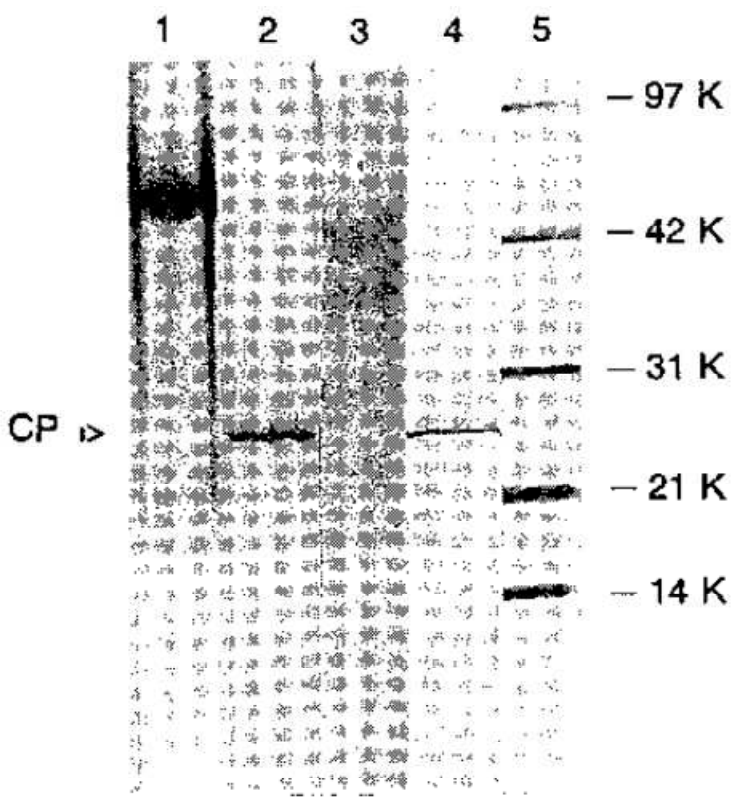


Fig. 6. Immunoelectrophoretic transfer of CMV proteins from 15% polyacrylamide gel onto nitrocellulose sheets. Lanes 1 and 2, proteins of healthy plant and purified CMV respectively, stained with Coomassie Blue. Lane 3 and 4, proteins of healthy plant and purified CMV respectively, after immunostaining. Lane 5, protein molecular weight markers.

Fig. 6 shows the immunodetected protein pattern of purifies CMV. From this pattern, a unique band of 24 K was observed. This virus-specific band showed a molecular weight lower than the molecular weight reported for the coat protein of CMV (9, 11).

## DISCUSSION

Two distinct aphid-style-borne transmissible viruses were associated with a severe mosaic disease in field melons in Costa Rica. Based upon the type of induced cellular inclusions, morphology and size of particles, inmunodifusion and immunoelectrophoretic transfer of protein results, the melon viruses were identified as PRSV and CMV.

The observation of different types of cytoplasmic inclusions (Fig. 2a and Fig. 2b) was the first evidence of single and mixed infections of potyvirus and cucumovirus associated with the severe mosaic showed in Fig. 1.

Partially purified, filamentous particles exhibit the typical morphology and size for potyvirus. The normal length of 781.5 nm for PRSV obtained in this work was

slightly different from the normal length reported for WMV-I by Milne and Grogan (17). The authors indicated that a number of variables could affect normal length determination of long flexuous rod-shaped viruses, and they therefore concluded that differences of 30 nm were within the range of experimental error with the electron microscopy techniques.

The isometrical particles of 29 nm observed in Fig. 3b were similar to those reported for CMV by Francki *et al*. (10).

The immunodiffusion results identified PRSV as the potyvirus and CMV as the cucumovirus involved in this disease.

Proteins from several potyviruses have been found to migrate as two bands on SDS-PAGE with apparent molecular weights of 32 - 34 K (23). The coat protein pattern of PRSV in this report revealed two bands with apparent molecular weights of 33 - 36 K by policlonal and monoclonal antibodies (Fig. 5). There have been numerous reports on the heterogeneity of the coat protein of potyviruses (23).

The CMV capside protein of 24 K molecular weight here described is slighdy different from some other isolates reported (9, 11) Rybicki and Von Wechmar (22) have reported apparent coat protein size differences between different CMV isolates.

Although CMV and PRSV have been reported previously in many countries around the world (5, 6, 7, 8, 13, 16, 18, 19), this is the first time they are reported in melons in Costa Rica.

Because of the widespread occurrence of CMV and PRSV and the severe symptoms observed associated with them, these viruses may become a mayor threat to commercial melon fields in Costa Rica. Current studies are concerned with the epidemiology of these viruses in order to design control strategies for these diseases.

Due to the similar symptomatology observed either in CMV, PRESV and mixed infected plants, the identification based on symptoms was unreliable.

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