

## MUNGBEAN YELLOW MOSAIC VIRUS ISOLATED FROM MUNGBEAN IN THAILAND<sup>1)</sup>

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### Abstract

The whitefly-borne mungbean yellow mosaic virus (MYMV) in Thailand was transmitted by mechanical inoculation. Among the several buffers used in attempted transmissions, 0.1 M potassium or sodium phosphate, pH 7.8, gave the highest transmission rates. The optimal incubation temperatures for symptom expression ranged from 25 to 30°C in the growth chamber or 30°C in the daytime and 20°C at night in the greenhouse. Host range of MYMV was limited to seven plant species in the family Leguminosae. Determinations of the stability of the virus in plant sap gave the following results: thermal inactivation point of 40–50°C for 10 min, dilution end point of 10<sup>-2</sup>–10<sup>-3</sup>, and longevity in vitro of 1–2 days at 20°C. Purified virus preparations had an ultraviolet light absorption spectrum typical of that of nucleoprotein with a A<sub>260</sub>/A<sub>280</sub> value of about 1.3–1.4. Purified preparations and leaf-dip samples contained geminate particles about 18 × 30 nm in size. Infectivity was associated with the presence of purified virus particles. In thin sections, the virus particles were isometric, about 15–20 nm in diameter, and they often formed loose aggregates that sometimes almost filled completely the nucleus of infected phloem cells. Mungbean infected by whitefly transmission or by grafting showed hypertrophied nucleoli, aggregates of virus particles, and fibrillar bodies in the nuclei of phloem cells as early as 2 days before symptom appearance. In vacuoles or lumens of the partially or fully differentiated infected sieve elements, virus particles occasionally formed aggregates having a double cylindrical arrangement of particles. No virus particles were detected in tissues other than the phloem of infected plants or in any tissues of comparable healthy plants.

### 1. Introduction

In early September 1977, the first report on the occurrence of mungbean yellow mosaic disease (MYMD) outbreak in Thailand was received from Kamphaeng Phet Province in northern Thailand (13). The disease also occurred in five other nearby

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1) Parts of the results on mungbean yellow mosaic virus were reproduced from *Phytopathology* 71:41–44, 1981 and *Plant Disease* 67:801–804, 1983 with the permission of the American Phytopathological Society (September 13, 1983).

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provinces. The MYMD caused almost total yield loss in infected mungbean (*Vigna radiata* (L.) Wilcz.) plants in the field. Laboratory tests indicated that the causal agent of the disease was transmitted by the tobacco whitefly, *Bemisia tabaci* Genn. (13). Prior to that report, a similar whitefly-borne disease had been observed in India (7). The disease in India was very severe in blackgram (*V. mungo* (L.) Hepp.) and caused as much as 100% yield loss when plants were infected at the seedling stage (8). Despite the severity of the MYMD in both countries, very little was known previously about the nature of the causal agent except that it was whitefly-borne and was not transmissible by mechanical inoculation (8). Isolation and characterization of the causal agent of MYMD have not been reported.

In this paper, we report the mechanical transmission, purification and ultrastructural studies of mungbean yellow mosaic virus (MYMV) in Thailand and describe some of the properties indicating that MYMV is a member of the geminivirus group.

## 2. Materials and methods

**1) Virus source, maintenance, and mechanical inoculation** Mungbean plants showing yellow mosaic symptoms were collected from fields in Kamphaeng Phet Province in northern Thailand. The virus was maintained in mungbean plants by whitefly and graft transmission in greenhouse at Tsukuba, Japan, or in an insect-proof house at Bangkok, Thailand. Seedlings of mungbean used for mechanical inoculation tests were 5–7 days old. All the inocula were prepared by grinding systemically infected young mungbean leaves in buffers (about 4 ml/g tissue) with a chilled mortar and pestle. Inoculations were performed by rubbing Carborundum-dusted primary leaves of the test plants with cotton wool soaked in the homogenate. Various buffers (potassium phosphate, sodium phosphate, borate, and Tris-HCl) with molarities of 0.05, 0.1, and 0.2, as well as 0.1 M potassium phosphate buffer with pH values of 4.5, 5.0, 6.0, 6.5, 7.0, 7.5, 7.8, 8.0, 8.5, 9.0, and 9.5 were used in the transmission studies.

The effect of the incubation temperature on symptom expression of inoculated plant was also tested at 15, 20, 25, 30, and 35°C in a growth chamber or at 30°C in the daytime and 20°C at night in a greenhouse. On this occasion, the inoculum was ground in 0.1 M potassium phosphate, pH 7.8, containing 1 mM KCN and inoculated to 30 test plants. The combination that gave maximum transmission rates was used in subsequent experiments.

**2) Host range and stability in sap** Host range of MYMV was determined by mechanical inoculation of 26 plant species belonging to six families. Young seedlings were used in these trials and were inoculated under optimal conditions for MYMV transmission. Inoculated plants were assayed by back-inoculation to mungbean seedlings 24 days after inoculation. In sap extracted from infected mungbean leaves, the thermal inactivation point (TIP), dilution end point (DEP), and longevity in vitro of the virus were determined using mungbean seedlings as test plants. The sap for TIP and LIV tests was diluted fourfold in 0.1 M sodium phosphate buffer, pH 7.8.

**3) Virus purification** Systemically infected leaves of French bean (*Phaseolus vulgaris* L. 'Top Crop') were harvested about 2 wk after mechanical inoculation. Healthy leaves of Top Crop bean were used as controls. Frozen leaves were

homogenized with a Waring Blender in 0.1 M potassium buffer, pH 7.8, containing 0.1% thioglycolic acid, 0.01 M sodium diethyldithiocarbamate, and 1 m M sodium ethylenediaminetetraacetate (2 ml/g tissue). The extract was clarified by adding one-half volume of chloroform and stirring at 4°C for 30 min. The emulsion was broken by centrifugation at 5,000 g for 10 min, and the aqueous phase was recovered.

Polyethylene glycol (PEG: mol wt 6,000) and sodium chloride were added to the aqueous phase to give a final concentration of 6% and 0.2 M, respectively. After stirring at 4°C for 2 hr, the mixture was centrifuged at 15,000 g for 30 min and the precipitates were dissolved in 0.1 M potassium phosphate buffer, pH 7.8, and clarified by centrifugation (9,000 g for 10 min) before being subjected to ultracentrifugation at 125,000 g for 90 min. The pellets were resuspended in potassium phosphate containing 6% PEG and 0.2 M NaCl. The resuspended pellets were layered onto PEG discontinuous reverse solubility gradients prepared by layering 12.5 ml of 40% sucrose and then another 12.5 ml of 10% sucrose containing 4% PEG and 0.2 M NaCl into each tube. About 2 ml of the sample was layered onto each gradient and centrifuged in a Hitachi RPS 25 swinging rotor at 12,000 rpm for 30 min.

The opaque band located at the interface between sucrose layers containing 4 and 0% PEG was recovered and concentrated by ultracentrifugation as before. Resuspended pellets were subjected to sucrose density gradient centrifugation in a Hitachi RPS 27-2 swinging rotor at 26,000 rpm for 3 hr using 10–40% linear sucrose gradients. After centrifugation, gradients were analyzed and fractionated by an ISCO Model 640 density gradient fractionator coupled with an ISCO Model UA-5 absorbance monitor. Ultraviolet light (254 nm) absorbing fractions were collected, pooled, and concentrated by ultracentrifugation as before and used for electron microscopy and absorbance spectrum analysis. The corresponding fractions from a sister gradient were also collected similarly and used for infectivity assays by mechanical inoculation to mungbean seedlings.

**4) Electron microscopy** Leaf-dip and purified virus samples for electron microscopy were mounted on collodion-carbon-coated grids and stained with 2% sodium phosphotungstate (PTA), pH 3.5, or 2% uranyl acetate. Observations were made with a Hitachi Model H300 or H500 electron microscope.

Mungbean leaf samples for thin sectioning were collected from inoculated plants 5, 7, 9, 11, 13, 15, and 18 days after inoculation either by whitefly or by grafting. Pieces of the infected leaves were fixed with 4% glutaraldehyde at 5°C for 1.5 hr, and were post-fixed with 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.5) at 5°C for 5 hr. After washing and dehydration, they were embedded in a mixture of low-viscosity epoxy resin (12). Thin sections were cut with a glass knife in a Porter-Blum Model MT 2B or a LKB 8800 Ultratome. They were double-stained with uranyl acetate and lead citrate before observation. Leaf samples from noninoculated mungbean plants at comparable age were similarly processed and served as controls.

### 3. Results

#### 1) Mechanical inoculation

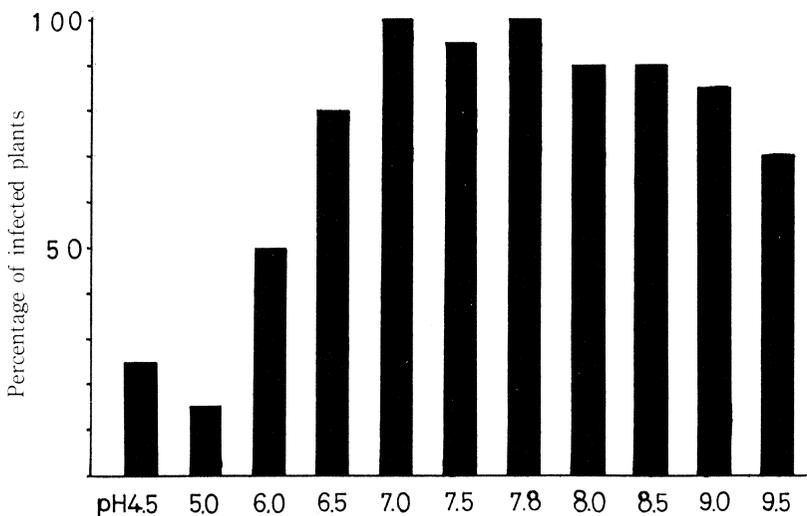
In preliminary experiments, MYMV could not be transmitted by mechanical inoculation. In later trials, however, the virus could be transmitted using conventional inoculation techniques. Among the several buffers used in attempted transmissions,

**Table 1. Effect of extraction buffer on the infectivity of mungbean yellow mosaic virus**

Buffers (pH 7.8)	Infectivity at buffer molarities of		
	0.05 M	0.1 M	0.2 M
Potassium phosphate	9 <sup>a)</sup>	9 <sup>a)</sup> (15) <sup>b)</sup>	0 <sup>a)</sup>
Sodium phosphate	3	9 (15)	0
Borate	5	8 (9)	2
Tris-HCl	5	0 (2)	5

a) Indicates the number of plants infected per 10 mungbean seedlings inoculated.

b) Figures in parentheses are the number of plants infected per 15 mungbean seedlings inoculated (each buffer containing 0.001 M KCN).



**Fig. 1. Infectivity of mungbean yellow mosaic virus in young mungbean leaf sap prepared in 0.1 M potassium phosphate buffer at different pH values. Twenty mungbean plants were inoculated in each case.**

0.1 M potassium or sodium phosphate, pH 7.8, gave transmission rates as high as 90–100% (Table 1). In potassium phosphate buffer at pH 4.5, 5.0, 6.0, 6.5, 7.0, 7.5, 7.8, 8.0, 8.5, 9.0, and 9.5, the percentages of mungbean seedlings with yellow mosaic symptoms were 25, 15, 50, 80, 100, 95, 100, 90, 85, and 70%, respectively (Fig. 1). The optimum pH values of phosphate buffer for transmission ranged between 7.0 and 7.8. At 15, 20, 25, 30, and 35°C in the growth chamber, the percentages of transmission were 0, 80, 87, 93, and 80%, respectively, and 97% in the greenhouse, where the temperature varied from 20°C minimum at night to 30°C maximum in the daytime. Although no symptoms appeared at 15°C, 70% of the test plants showed symptoms when maintained at 15°C for 23 days and then kept in the greenhouse for 22 days.

## 2) Host range, symptomatology, and stability in sap

Among the 26 plant species belonging to six families used in the mechanical inoculation tests, only seven species of the family Leguminosae were infected with MYMV. Symptoms consisted mainly of yellow mosaic or leaf curl. In systemically infected leaves of azuki bean (*Phaseolus angularis*), blackgram, mungbean (Fig. 2A, B), and soybean (*Glycine max*) (Fig. 2C), vein yellowing along the veinlets appeared at the early stages, then developed into severe yellow mosaic symptoms. First emerging trifoliolate leaves of mungbean showed severe downward curling.

In infected French bean (*P. vulgaris*), trifoliolate leaves showed downward curling without yellow mosaic symptoms (Fig. 2D). In infected jack bean (*Canavalia ensiformis*) and lima bean (*P. lunatus*), vein yellowing developed in leaflets, followed by mild downward curling. These plants did not show severe yellow mosaic symptoms in the advanced stages of infection. Back-inoculation to mungbean seedlings from infected plants resulted in yellow mosaic symptoms in test plants. MYMV did not infect *Tetragonia expansa* (Aizoaceae), *Gomphrena globosa* (Amaranthaceae), *Chenopodium amaranticolor*, *C. quinoa* (Chenopodiaceae), *Cucumis sativus* (Cucurbitaceae), *Arachis hypogaea*, *Cassia occidentalis*, *Cassia tora*, *Centrosema pubescens*, *Dolichos lablab*, *Pisum sativum*, *Vicia faba*, *V. sesquipedalis*, *V. unguiculata* (Leguminosae), and *Datura stramonium*, *Lycopersicon esculentum*, *Nicotiana glutinosa*,

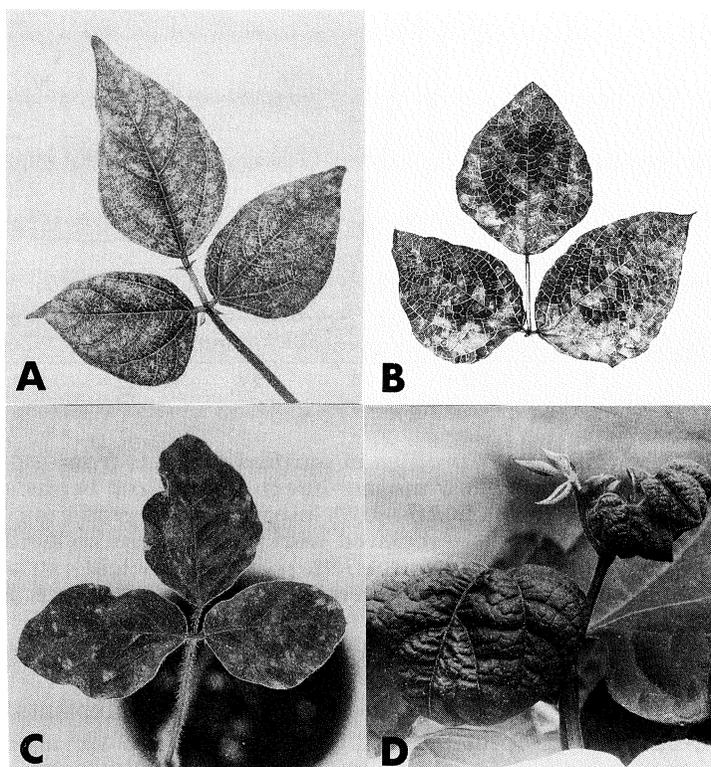


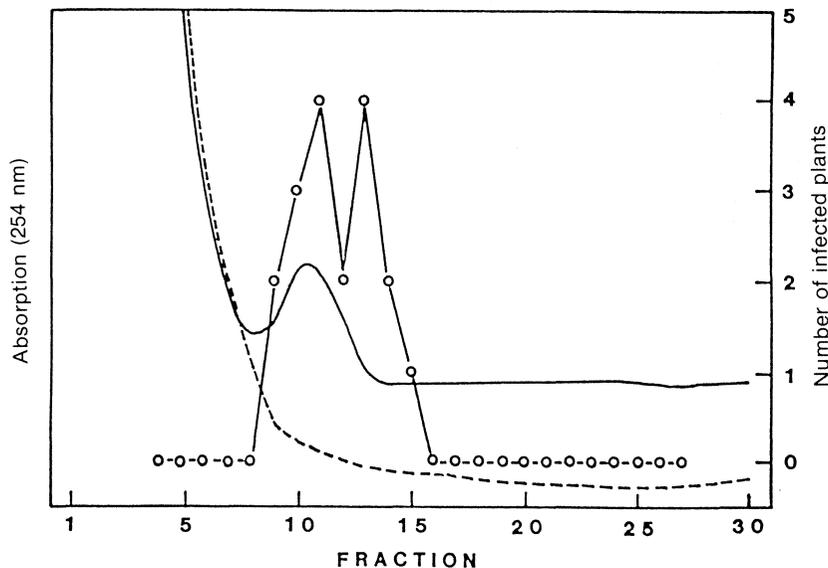
Fig. 2. Symptoms of infection with mungbean yellow mosaic virus. (A): Vein yellowing in mungbean. (B): Yellow mosaic in mungbean. (C): Yellow mosaic in soybean. (D): Severe downward curling in Top Crop bean.

*N. tabacum*, and *Petunia hybrida* (Solanaceae).

In sap extracted from infected mungbean leaves, the virus showed a TIP of 40–50°C for 10 min, DEP between  $10^{-2}$  and  $10^{-3}$ , and LIV of 1 or 2 days at 20°C.

### 3) Virus purification and electron microscopy

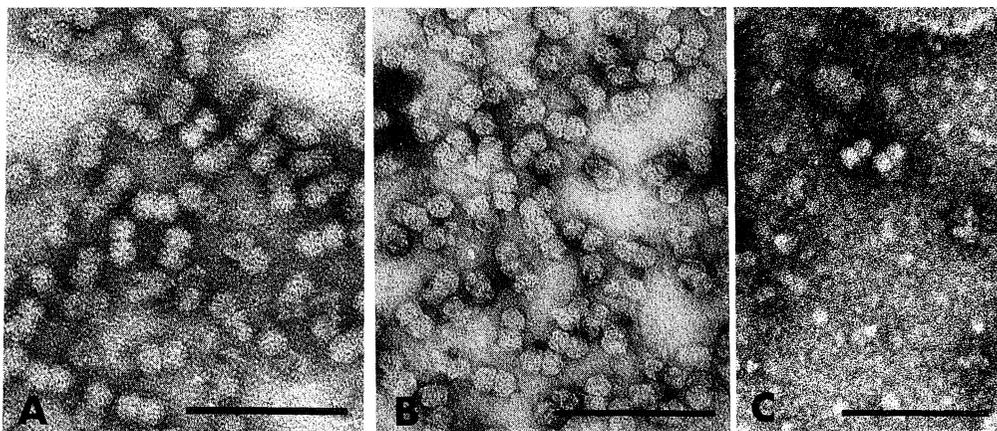
After centrifugation in PEG reverse concentration gradients, two major bands were observed, one located slightly below the meniscus and the other, which was opaque, located at the interface between the two layers of sucrose containing 4 and 0% PEG. Sucrose density gradient centrifugation and electron microscopy revealed that the first band contained predominantly phytoferritins, whereas the second band contained geminate particles. The geminate particles sedimented in 10–40% linear sucrose gradient as a single band located at fractions 9–15 (Fig. 3). When employed for mechanical inoculation, these fractions proved infective in five mungbean seedlings (Fig. 3).



**Fig. 3. Sedimentation profiles of purified extracts from mungbean yellow mosaic-infected Top Crop beans and healthy beans in 10–40% linear sucrose gradients and infectivity associated with the fractions collected. Sedimentation from the left. --- = Healthy leaf extract absorbance, — = infected leaf extract absorbance, and ○—○ = infectivity (each fraction collected was inoculated to five mungbean seedlings).**

Symptoms obtained were similar to those shown by mungbean plants infected with MYMV (Fig. 2A, B). The size of the purified geminate particles was about  $18 \times 30$  nm (Fig. 4A, B). Electron microscopy of leaf-dip samples prepared from young mungbean leaf tissue infected with MYMV also revealed particles of similar size and shape (Fig. 4C). The corresponding fractions from healthy tissues treated similarly failed to show a peak and geminate particles. The preparations with geminate particles had an

ultraviolet light absorption spectrum characteristic of that of nucleoprotein with a  $A_{260}/A_{280}$  value of 1.3-1.4 (Fig. 5). Assuming the extinction coefficient ( $E_{cm}^{1\%}$  260 nm) was 7.7 as for the geminate particles of bean golden mosaic virus (3), the yield of the geminate particles associated with MYMV was below 1 mg/kg tissue.

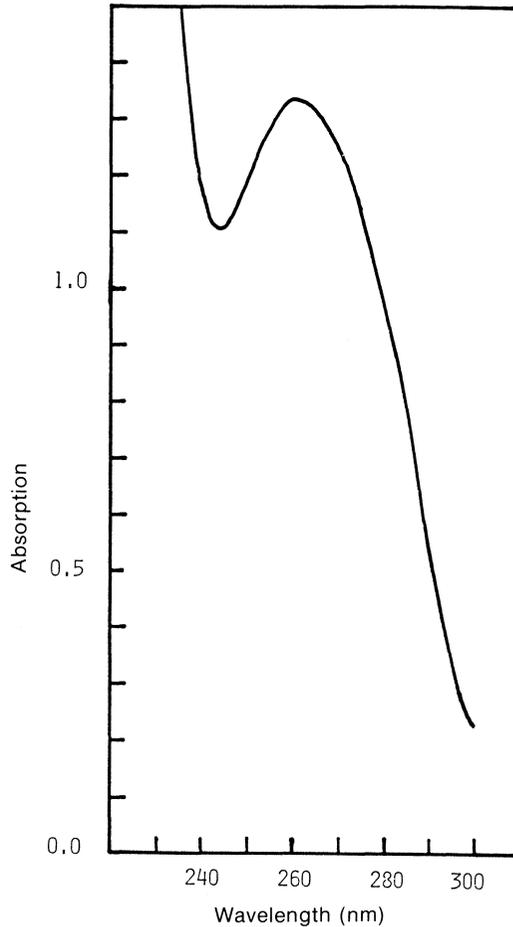


**Fig. 4.** Electron micrographs of mungbean yellow mosaic virus particles. (A and B): Purified virus preparations negatively stained with 2% sodium phosphotungstate (PTA), pH 3.5, and 2% uranyl acetate, respectively. (C): Infected mungbean leaf-dip samples negatively stained with 2% PTA, pH 3.5. Bar = 100 nm.

#### 4) *Thin sectioning*

The first ultrastructural changes observed were in phloem tissues sampled 7 days after inoculation (2 days prior to symptom appearance). The nucleoli of some phloem cells from such symptomless tissues were hypertrophied. Loose aggregates of MYMV particles were observed in the nucleus. The aggregates varied in size and shape, and sometimes occupied almost the total nuclear volume (Fig. 6A, B). The aggregates of virus particles were more frequently observed in the nucleus as the time after inoculation progressed. These aggregates were found in nuclei whether the nucleoli were present or absent. In nuclei containing nucleoli and aggregates of virus particles, the size of the nucleolus was usually smaller than that of the aggregate (Fig. 7A). The aggregates were highly electron-dense and the outlines of small virus particles were evident, in comparison to the ill-defined contents of the nucleolus (Fig. 7B). The diameter of the virus particles was 15-20 nm. Fibrillar bodies (usually one or two per nucleus) with the shape of either a solid circle (Fig. 8A) or ring (Fig. 8B), depending upon the orientation of sectioning, were occasionally observed along the edge of the aggregates of virus particles or scattered in the nucleoplasm.

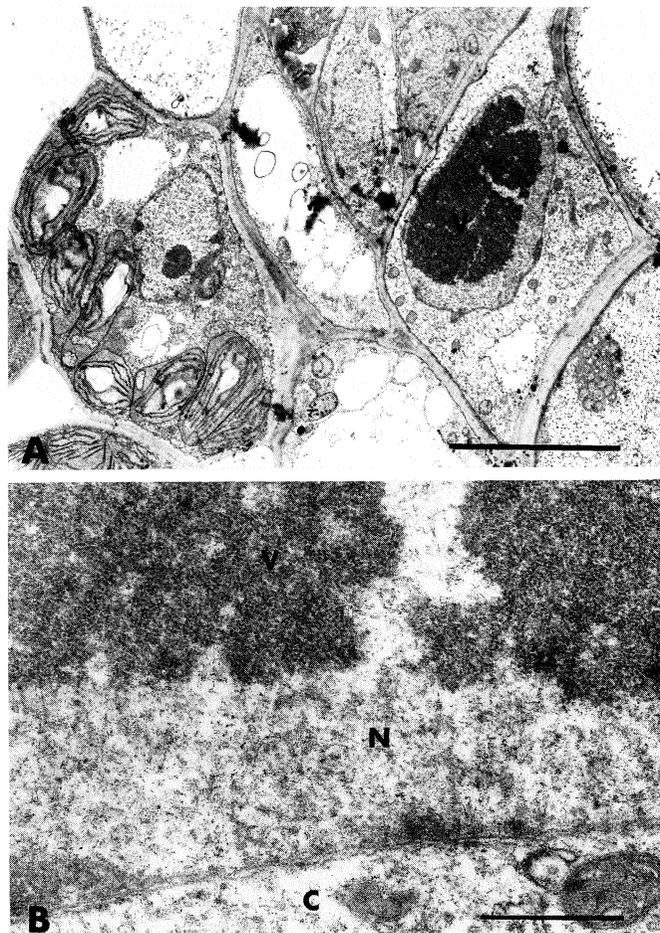
Virus particles were observed in the vacuoles or lumens of partially or fully differentiated sieve elements; the particles were either scattered in the sieve tubes or arranged into five loose or paracrystalline aggregates (Fig. 9A). In addition, aggregates of virus particles having a double cylindrical arrangement were observed (Fig. 9B). The double cylindrical aggregates consisted of rows of virus particles with indefinite numbers of particles to give small cylinders, each having five rows of particles. About



**Fig. 5. Ultraviolet light absorption spectrum of purified mungbean yellow mosaic virus preparations.**

seven or eight of these small cylinders were in turn arranged to form a large cylinder. The number of the small cylinders giving rise to each large cylinder could not be precisely determined since the side-by-side arrangement of the neighboring rows of particles was slightly twisted, so that some small cylinders were cut tangentially in the plane perpendicular to the cylinder axis.

Although hypertrophied nucleoli and virus particles and their aggregates were present in tissues sampled 2 days, but not 4 days, before symptom appearance, they were more frequently observed in tissues sampled several days after symptom appearance. Tissues sampled 7 and 10 days after symptom appearance often were necrotic and virus particles were rarely observed. No differences in ultrastructural changes were detected between tissues infected by whitefly transmission or grafting. No ultrastructural changes or virus particles were observed in comparable healthy tissues.



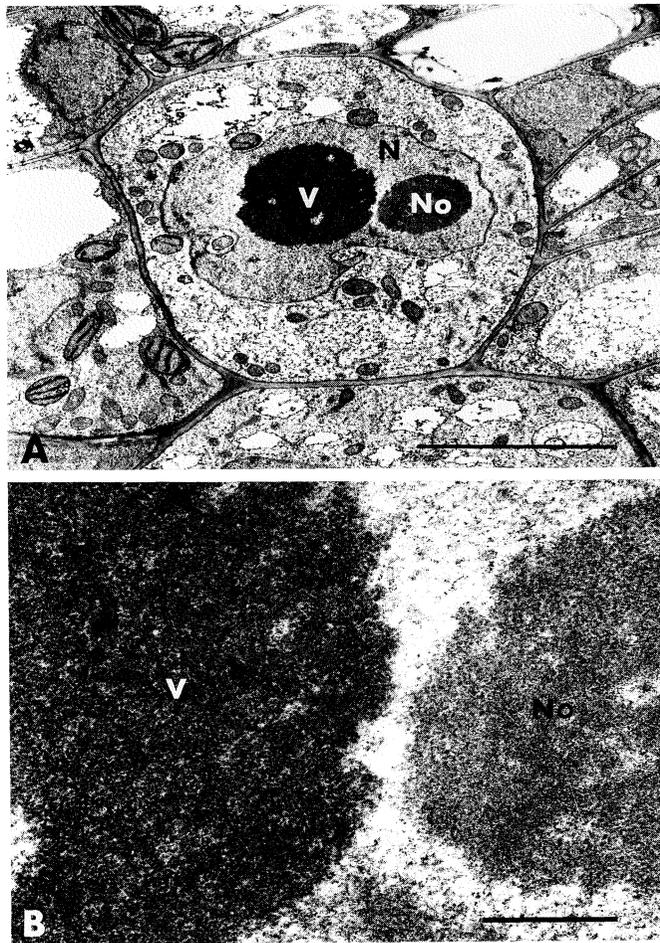
**Fig. 6. (A): The aggregate of virus particles (V) occupying almost the total nuclear volume of the phloem cell of mungbean yellow mosaic virus-infected mungbean leaf. Bar = 5  $\mu$ m. (B): Enlarged view of A. Individual virus particles (V) can be seen in the nucleus (N). C = cytoplasm. Bar = 0.5  $\mu$ m.**

#### 4. Discussion

The geminate particles found in the leaf-dip preparations from mungbean plants infected with MYMV together with the infectivity of the particles revealed in the purification experiments clearly indicate for the first time that the causal agent of MYMD is a geminivirus. These results confirm the original contention based on ultrastructural studies that the MYMD was caused by such a virus in this report. Although early reports both from India (8) and Thailand (13) indicated that MYMV was not transmissible by mechanical inoculation, attempts in our subsequent trials were successful. MYMV can be mechanically transmitted by conventional inoculation techniques at a rate as high as 100% with or without a reducing agent in the phosphate buffers. The whitefly was able to transmit the pathogen to healthy

mungbean, soybean, and Top Crop bean from mungbean infected with MYMV by mechanical inoculation (Y. Honda, unpublished). MYMV particles are relatively stable compared with those of many other whitefly-transmitted geminiviruses because they can be seen under the electron microscope without prior fixation with aldehydes. Geminate particles were detectable by the leaf-dip method after negative staining with PTA; hence, it is possible to use this technique for preliminary diagnosis of the disease.

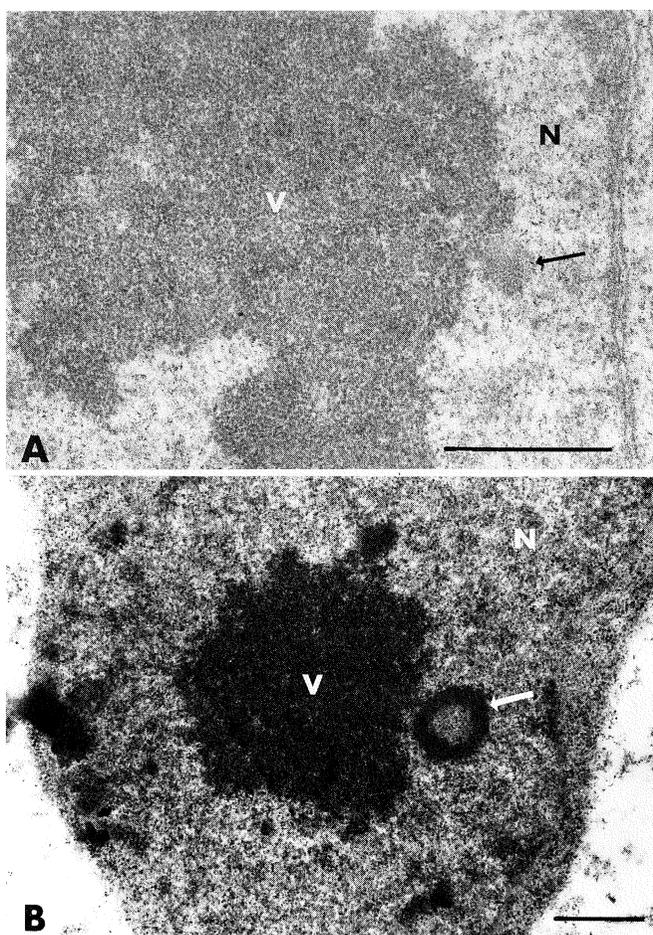
The yellow mosaic disease reported from India was more severe in blackgram than in mungbean (14); however, the disease in Thailand was more common in mungbean and was seldom observed in blackgram under natural conditions. Our inoculation



**Fig. 7.** (A): The nucleolus (No) and the aggregate of virus particles (V) in the nucleus (N) of the phloem cell of the mungbean yellow mosaic virus-infected mungbean leaf. Bar = 5  $\mu\text{m}$ . (B): Enlargement of A. The aggregate of virus particles (V) was highly electron-dense and the outline of small spherical particles was evident, in comparison to the contents of the nucleolus (No). Bar = 0.5  $\mu\text{m}$ .

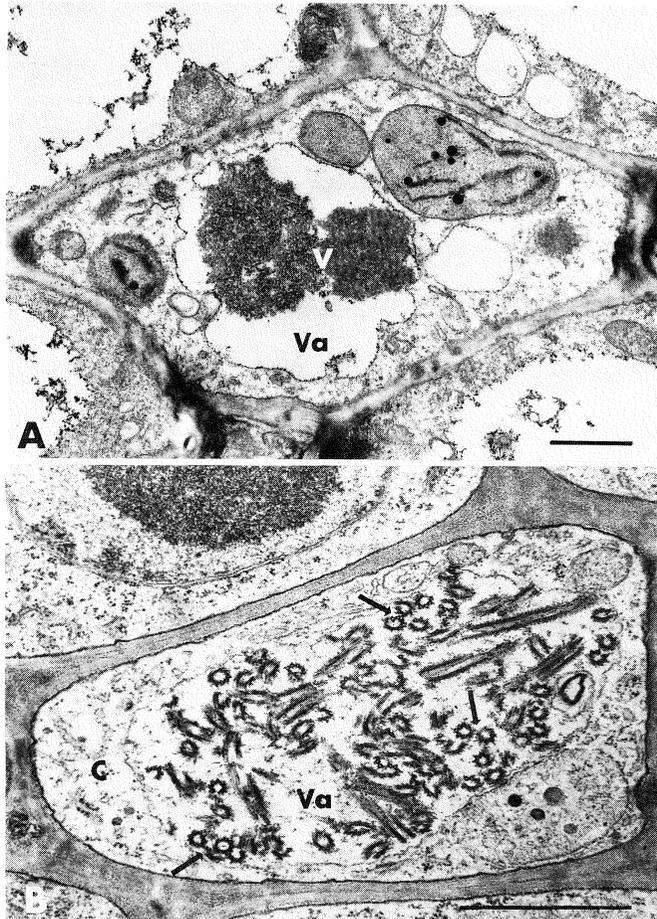
studies in the greenhouse revealed that MYMV could infect blackgram but that the transmission rates in blackgram were lower than in mungbean. The virus reported from India also has a wider host range including plant species such as *Brachiaria ramosa* (Gramineae) and *Eclipta alba* (Compositae) (9). The host range of MYMV in Thailand appears to be restricted to plants in the family Leguminosae.

By comparing the host range of MYMV in Thailand with that of bean golden mosaic virus (BGMV) in Puerto Rico, it was found that BGMV could not be transmitted by inoculation with sap or graft to blackgram, mungbean and soybean from Top Crop bean (Y. Honda, unpublished). In contrast, MYMV could be transmitted to them. Symptoms incited by BGMV in Top Crop bean (3) were different from those caused by MYMV (Fig. 2D).



**Fig. 8.** Fibrillar bodies in the nucleus of the phloem cell of the mungbean yellow mosaic virus-infected mungbean leaf. (A): The fibrillar body (arrow) with the shape of the solid circle associated with the aggregate of virus particles (V) in the nucleus (N). Bar = 0.5  $\mu\text{m}$ . (B): The fibrillar body (arrow) with ringlike appearance associated with the aggregate of virus particles (V) in the nucleus (N). Bar = 0.5  $\mu\text{m}$ .

Our findings of ultrastructural changes and MYMV particles in phloem cells of mungbean plants are the first direct evidence indicating that the yellow mosaic disease of mungbean in Thailand was caused by a virus. The ultrastructural abnormalities in infected phloem cell nuclei (ie, hypertrophied nucleoli, fibrillar bodies, and aggregates of virus particles) were similar to those reported for several other diseases induced by whitefly-transmitted geminiviruses (5, 6, 10, 11). However, we did not detect segregation of nucleolar contents into granular and fibrillar regions as reported for other whitefly-transmitted viruses (5, 6, 11). Although the origin of the fibrillar bodies is unclear, it seems that they are a consequence of the disease. The shape of the fibrillar bodies found in our experiments is very similar to the fibrillar rings which are found in nuclei of BGMV-infected bean cells (6). Kim *et al.* (6) reported



**Fig. 9.** (A): Paracrystalline and loose aggregates of virus particles (V) in the vacuole (Va) of the partially differentiated sieve element of the mungbean yellow mosaic virus-infected mungbean leaf. Bar = 1  $\mu$ m. (B): Double cylindrical aggregates (arrows) of virus particles in the vacuole (Va) of the sieve element of the mungbean yellow mosaic virus-infected mungbean leaf. C = cytoplasm. Bar = 1  $\mu$ m.

cytochemical evidence indicating that fibrillar rings in BGMV-infected bean cells are deoxyribonucleoprotein which was different from the ribonucleoprotein material of nucleolar contents. These results led them to conclude that the fibrillar rings were not modifications of preexisting nucleoli but were formed after virus infection (6).

The double cylindrical aggregates of virus particles found in our experiments are somewhat similar to the rod-shaped aggregates of paired particles of beet curly top virus, chloris striate mosaic virus (CSMV) and tobacco leaf curl virus (TLCV) (1, 2, 10). However, the rod-shaped aggregates of paired particles were less organized than the double cylindrical aggregates of MYMV particles and were found only in the nucleoplasm of the infected cells, except for CSMV aggregates which were also present in the cytoplasm; other geminiviruses have not been reported in the cytoplasm of infected cells. We observed the double cylindrical aggregates of MYMV particles in tissues sampled as early as the first day and as late as the fifth day after symptom appearance.

Considering the results of the ultrastructural changes induced by the infection of BGMV and TLCV together with the findings in our experiments, we agree with Kim and Flores (5) and Kim *et al.* (6) that ultrastructural aspects of host cells may possibly be used as a criterion for the diagnosis of diseases caused by whitefly-transmitted geminiviruses.

We characterized the nucleic acid of MYMV. Nucleic acid isolated from MYMV particles was identified as circular single-stranded DNA with a molecular weight of  $8.0 \times 10^5$  (in preparation) (4).

### Acknowledgments

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