OCCURRENCE OF BLACKGRAM MOTTLE VIRUS ON MUNGBEAN IN INDONESIA AND SEED TRANSMISSION OF THE VIRUS

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Abstract

Blackgram mottle virus (BMoV) was isolated from mungbean plants showing mottling and mosaic symptoms in Indonesia. The virus was transmitted by mechanical inoculation, the bean leaf beetle, *Colposcelis signata* and golden green minute leaf beetle, *Balisepta fulvipes* and through infected mungbean seeds. Purified virus preparations contained isometric particles with a diameter of about 28 nm and had an ultraviolet light absorption spectrum typical of those of nucleoprotein components with a A_{260}/A_{280} value of about 1.53. Indonesian and Thai BMoV showed reaction of identity in Ouchterlony double-diffusion tests using antiserum against Indonesian BMoV. Indonesian BMoV also reacted with antiserum against Indian BMoV, but did not react with antisera against some other beetle-borne viruses. Enzyme-linked immunosorbent assay (ELISA) test detected BMoV at concentrations as low as 50 ng/ml and was more sensitive than the mechanical inoculation test. Both ELISA and mechanical inoculation tests could easily detect BMoV at high concentrations from whole seeds, but at very low concentrations in seeds in which seed coats had been removed.

1. Introduction

In 1980, mungbean plants showing mottling and mosaic symptoms were collected at Cikeumeuh Experimental Station, Bogor, Indonesia. The preliminary studies showed that the causal virus consisted of isometric particles and induced symptoms on mungbean which were different from those of mungbean mosaic virus and bean yellow mosaic virus previously reported by Iwaki (4).

In 1968, blackgram mottle virus (BMoV)-infected blackgram, *Vigna mungo* L., was found near New Delhi, India (7). The virus was seed-borne in about 5% of infected blackgram. It was transmitted by the bean leaf beetle, *Cerotoma trifurcata* and the Mexican bean leaf beetle, *Epilachna varivestis*. BMoV was readily sap-transmissible and was restricted to legumes in host range. BMoV consisted of isometric particles ca. 28 nm in diameter with single-stranded RNA (8, 9). Reciprocal serological tests showed no relationships between BMoV and other members of the beetle-transmitted legume virus group (8, 9).

In 1979, BMoV was also reported on mungbean, blackgram and soybean in Thailand during the collaborative research project entitled "Studies on rice and legumes virus diseases in the tropics" (3). Thai BMoV was transmitted by the beetle, *Monolepta signata*. BMoV isolated from mungbean and soybean in Thailand showed reaction of identify in Ouchterlony double-diffusion tests using antiserum against BMoV

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previously reported from India (9), but did not react with antisera against four other beetle-borne viruses (3).

This report describes the results of host range, symptomatology, transmission, purification, electron microscopy and serological relationships of BMoV isolated from mungbean plants showing mottling and mosaic in Indonesia. We also describe the results of BMoV detection in mungbean seeds by enzyme-liked immunosorbent assay (ELISA) and infectivity test by mechanical inoculation.

2. Materials and methods

1) Virus source and maintenance The virus was originally recovered from naturally infected mungbean plants in the field, Bogor. Isolation of the virus was carried out through serial single-lesion transfers on *Phaseolus vulgaris* L. cv. Top Crop. The isolated virus was maintained on *P. vulgaris* L. cv. Yamashiro Kurosando by successive mechanical inoculations.

2) Host range and symptomatology Host range of the virus was determined by mechanical inoculations of diseases leaves extracted in 0.05 M phosphate buffer, pH 7.2, to 21 plant species in six families in the greenhouse.

3) Insect transmission Virus-free cowpea aphids, *Aphis craccivora* Koch, were allowed to feed on diseased mungbean plants for 15 min, 30 min, 1 hr, 3 hr and 20 hr and followed by an inoculation access of 18 hr on healthy mungbean plants. Bean leaf beetle, *Colposcelis signata* Motschulsky and golden green minute leaf beetle, *Basilepta fulvipes* Motschulsky, were collected and maintained on white clover and mugwort plants. The beetles were allowed an acquisition access of one day on infected mungbean plants in tubular plastic cages followed by an inoculation access of one day on healthy mungbean plants. Three beetles per plant were used for the transmission tests.

4) Seed transmission Seed samples were harvested from BMoV-infected M7A, TM108, MB129 and MV436 mungbean plants in Indonesia. Each group of 200 mature seeds was individually grown in earthern pots containing steam-sterilized soil in an insect proof greenhouse at Tsukuba, Japan to determine the percentage of seed transmission. Seed-borne infection was evaluated the presence of BMoV symptoms on trifoliolate leaves.

5) Purification and electron microscopy The virus was purified by extracting sap from Yamashiro Kurosando bean leaves at 14 days after inoculation in 0.2 M sodium phosphate-buffer, pH 7.2, containing ˆ.1% thioglycolic acid (2 ml buffer/g tissue). The sap was clarified by addition of chloroform of 2 : 1 (v/v), stirred for 1 hr and centrifuged at 6,000 g for 10 min. The clarified sap was recovered from the aqueous phase and subjected to two cycles of differential centrifugation (ultra-centrifugation and high-speed centrifugation at 100,000 g for 1 hr and 10,000 g for 10 min, respectively). The pellets obtained after the last ultracentrifugation were resuspended in phosphate buffer and analyzed by density gradient centrifugation in a Hitachi RPS 27-2 rotor, 26,000 rpm for 3 hr, through 10-40% linear sucrose density gradient. Ultraviolet light (254 nm) absorbing fractions were collected and concentrated by ultracentrifugation as described above. Virus pellets were

resuspended in 0.01 M phosphate buffer, pH 7.2. Purified virus preparations were mounted on a carbon-stabilized-collodion coated grid and stained with neutral 2% phosphotungstic acid for examination in a Hitachi Model H500 electron microscope.

6) Thin sectioning Small pieces of BMoV-infected mungbean leaves were examined under an electron microscope. Procedure for thin sectioning was followed that for mungbean yellow mosaic disease (10).

7) Serological test Rabbits were given each week or at two week intervals intravenous or intramuscular injections of purified Indonesian BMoV to a total of 21 mg of virus. The titer of the antiserum was determined by mixed flocculation test and Ouchterlony double-diffusion test. Serological relationships between the Indonesian and Thai BMoV (3) were determined by Ouchterlony double-diffusion test using antiserum against the Indonesian BMoV. Reactions of Indonesian BMoV to antisera against the Indian BMoV (9) and other beetle-borne viruses, namely radish mosaic virus (RMV), southern bean mosaic virus (SBMV), squash mosaic virus (SqMV) and turnip yellow mosaic virus (TYMV) were also tested.

8) Detection of virus in mungbean seeds by ELISA Procedures for ELISA test closely followed those developed by Clark and Adams (1).

The γ-globulins from the antiserum to the Indonesian BMoV were purified by precipitation with ammonium sulfate, equilibration in half-strength phosphate buffered saline (PBS), pH 7.4, containing 0.02% sodium azide, and washing through DEAE cellulose in PBS. One mg of γ-globulin was conjugated with 2 mg of alkaline phosphatase (type VII, Sigma Chemical Co.) using 0.05% glutaraldehyde as the coupling agent and stored at 4°C with 1% bovine serum albumin after thorough dialysis in PBS.

Each well of a microtiter plate (Micro Elisa, Dynatech Ltd.) was precoated with 200 μl of nonlabelled γ-globulin in 0.05 M sodium carbonate, pH 9.6, incubated at 37°C for 2 hr and rinsed four times with PBS containing 0.05% Tween-20. Two hundred μl of crude extracts of virus-infected mungbean seed in PBS-Tween containing 2% polyvinyl pyrrolidone (PVP) and 0.2% egg albumin were added and incubated overnight at 6°C to react with the bound γ-globulin. After further rinsing, conjugated γ-globulin diluted 100- to 3,200-fold in PBS mixture was added to react with the bound antigen during 4 hr incubation at 37°C. Nonreacted conjugate was rinsed way. Specific antigen-antibody reactions were detected by adding 300 μl aliquots of freshly prepared *p*-nitrophenyl phosphate substrate at the rate of 1 mg per ml in 10% diethanolamine buffer, pH 9.8. Reactions were arrested after 2 hr by adding 50 μl of 3 M sodium hydroxide.

Assessment was made by visual inspection of the resulting yellow color from nitrophenol hydrolysis products, or by reading absorbances at 405 nm in a Hitachi 200-20 spectrophotometer.

Four varieties of mungbean seeds (M7A, MB129, MB436 and TM108) were examined by ELISA. Seeds were harvested from infected mungbean plants. Matured seeds were soaked overnight in distilled water and extracted in 1 ml of PBS-Tween containing 2% PVP and 0.2% egg albumin. For the examination of virus distribution in immature seeds, seed coats were removed before extraction in buffer. The crude extracts were squeezed through a cheesecloth and used for ELISA test. Healthy immature seeds were prepared for ELISA test as a control.

Mungbean seeds from the same batches were used for the infectivity test of BMoV. Infectivity test was assessed by mechanical inoculation to primary leaves of Top Crop bean.

3. Results

1) Host range and symptomatology

Among the 21 plant species in six families tested, 12 plant species in four families were infected with BMoV by mechanical inoculation (Table 1). All the leguminous plants except *Pisum sativum* and *Vicia faba* were infected with the virus. In the soybean cultivars Master Piece and Yamashiro Kurosando, chlorotic spots appeared on the inoculated leaves 3–4 days after inoculation followed by mosaic and leaf roll symptoms on the emerging trifoliolate leaves. The bean cultivars Top Crop and Honkintoki showed pinpoint necrotic lesions on the inoculated leaves without any systemic symptoms. They were used for assay species of BMoV.

BMoV induced necrotic lesions on inoculated leaves of the soybean cultivars Okuharawase and Shirotsurunoko from Japan without systemic infection. In contrast the soybean cultivar SJ 4 from Thailand showed necrotic lesions on the inoculated

Table 1. Host range of blackgram mottle virus isolated from mungbean in Indonesia

Family	Species	Symptoms[a)]	
		Inoculated leaves	Uninoculated leaves
Amaranthaceae	*Gomphrena globosa*	Lc	—
Chenopodiaceae	*Chenopodium amaranticolor*	Lc	—
	C. quinoa	Lc	—
Cucurbitaceae	*Cucumis sativus*	—	—
Leguminosae	*Arachis hypogaea*	1	—
	Glycine max 'Okuharawase'	Ln	—
	'Shirotsurunoko'	Ln	—
	'SJ4'	Ln	s
	Phaseolus angularis	Ln	—
	P. vulgaris 'Honkintoki'	Ln	—
	'Top Crop'	Ln	—
	'Master Piece'	Lc	M, LR
	'Yamashiro Kurosando'	Lc	M, LR
	Pisum sativum	—	—
	Vicia faba	—	—
	Vigna mungo	Ln	M
	V. radiata	Ln	M
	V. sesquipedalis	Lc	—
	V. unguiculata	Ln	—
Pedaliaceae	*Sesamum indicum*	Lc	—
Solanaceae	*Lycopersicon esculentum*	—	—
	Nicotiana clevelandii	—	—
	N. glutinosa	—	—
	N. tabacum 'Bright Yellow'	—	—
	Petunia hybrida	—	—
	Solanum melongena	—	—

a) Key to symptoms: 1 = symptomless local infection, Lc = chlorotic local lesions, Ln = necrotic local lesions, LR = leaf roll (downward), M = mosaic, s = symptomless systemic infection, — = no infection.

leaves and was systemically infected with BMoV without visible symptoms. We therefore concluded that the soybean reactions to BMoV infection depended upon the cultivars used.

Infected blackgram and mungbean plants showed chlorotic lesions with some necrotic specks on the inoculated leaves and mild mottling or mosaic symptoms appeared on systemically infected leaves.

BMoV induced only chlorotic lesions on the inoculated leaves of *Gomphrena globosa, Chenopodium amaranticolor, C. quinoa* and *Sesamum indicum.*

The virus did not infect six plant species in the Solanaceae.

2) Transmission by vectors

Transmission tests by insects showed that cowpea aphid, *Aphis craccivora,* could not transmit the virus. Bean leaf beetle, *Colposcelis signata* and golden green minute leaf beetle, *Basilepta fulvipes* were able to transmit the virus at the rates of 25% (1/4) and 89% (17/19) (infected plants/inoculated plants), respectively.

Further studies of virus transmission by *B. fulvipes* indicated that a high rate of virus transmission (85–100%) was obtained with 3 beetles per plant. Minimum acquisition access was 30 min and minimum inoculation access was 10 min. *B. fulvipes* could transmit BMoV efficiently for 3 days after feeding on the virus-infected plants.

3) Seed transmission

Mature seeds for seed transmission were harvested from infected mungbean plants. The level of transmission through seeds of TM108 and M7A mungbean was 1% and 1.5%, respectively (Table 3).

Table 3. Distribution of blackgram mottle virus in mungbean seeds and seed transmission

Variety of mungbean	Seed part	Virus detection by ELISA test [a] (%)	Virus detection by Infectivity test [b] (%)	Seed transmission Progeny symptoms [c] (%)
M7A	whole seed	99.0	90.0	1.5
	seed without seed coat	1.1	5.0	—
MB 129	whole seed	66.7	50.0	0.0
	seed without seed coat	—	5.0	—
MB 436	whole seed	25.0	25.0	0.0
	seed without seed coat	1.0	0.0	—
TM 108	whole seed	10.4	10.0	1.0
	seed without seed coat	2.1	5.0	—

a) 96 to 192 individual mature seeds were examined by ELISA test.
b) 20 individual mature seeds were inoculated to primary leaves of the bean cultivar Top Crop.
c) Progeny symptoms based upon a sandbench test for a separate 200 mature seed sample.
Symbol — = not tested.

4) Purification and electron microscopy

The Indonesian BMoV showed a single ultraviolet light (254 nm) absorbing zone in sucrose density gradient profiles. The purified virus had an ultraviolet light absorption spectrum typical of those of nucleoprotein components with a A_{260}/A_{280} ratio of about 1.53. By using the extinction coefficient ($E^{0.1\%}_{260nm}$) value of 5.0 reported for the BMoV from India (9), the yields of purified virus were about 70 mg/kg of bean tissue.

Electron microscopic examination of purified virus preparations revealed the

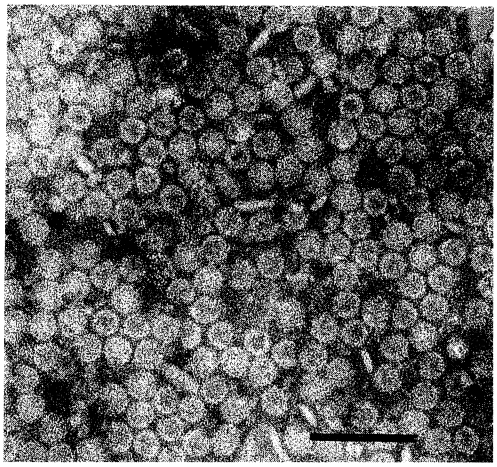

Fig. 1. Electron micrograph of purified blackgram mottle virus particles negatively stained with sodium phosphotungstate, pH 6.9. Bar = 100 nm.

presence of isometric virus particles approximately 28 nm in diameter (Fig. 1).

5) Thin sectioning

In thin sections of infected mungbean leaves, BMoV were observed in the cytoplasm of epidermal and mesophyll cells. The virus particles were distributed throughout the cytoplasm in crystalline arrays (Fig. 2A) and they were sometimes scattered through the central vacuole (Fig. 2A, B).

6) Serology

The antiserum against the Indonesian BMoV obtained from immunized rabbit had a titer of 1/160 in Ouchterlony double-diffusion tests and 1/1,280 in mixed flocculation tests. In Ouchterlony double-diffusion tests using the antiserum against the Indonesian BMoV, both of the Indonesian and Thai BMoV showed a reaction of identity (Fig. 3A). Indonesian BMoV reacted only with antisera against Indonesian and Indian BMoV, and did not react with antisera against RMV, SBMV, SqMV or TYMV (Fig. 3B).

7) Detection of virus in mungbean seeds by ELISA

Preliminary studies of ELISA established that the use of 2.5 μg of purified γ-globulin and enzyme-conjugated γ-globulin at a dilution of 1/800 gave the optimum

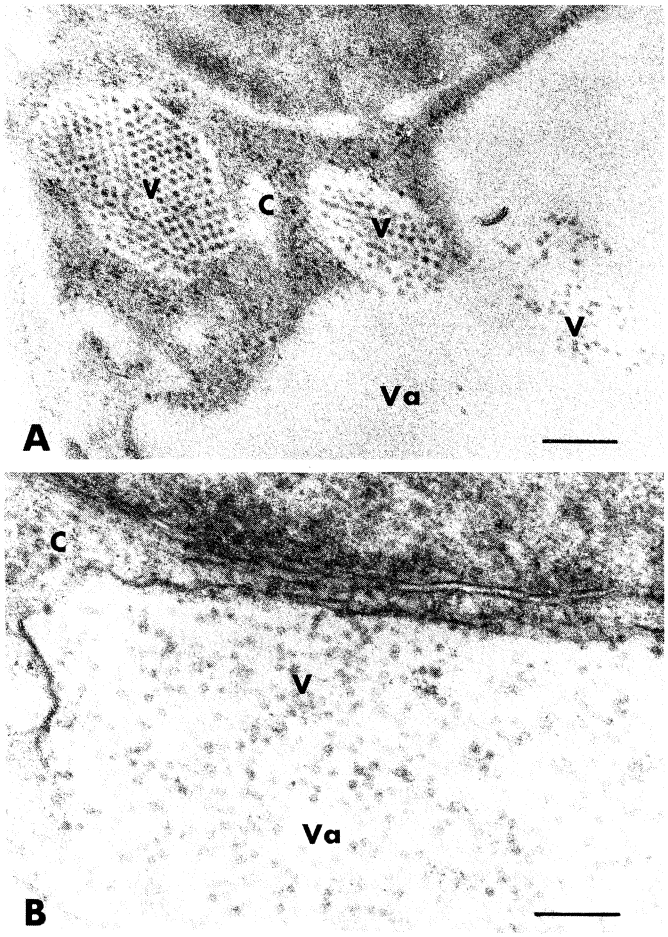

Fig. 2. Thin sections of blackgram mottle virus-infected mungbean leaves. (A): Spherical virus particles (V) observed in the cytoplasm (C) and the vacuole (Va) of the necrotic mesophyll cell. Bar = 200 nm. (B): Spherical virus particles scattered through the central vacuole (Va) of the mesophyll cell. C: cytoplasm. Bar = nm.

reaction for the coating and trapping of the BMoV. BMoV diluted in PBS could be detected at concentrations as low as 50 ng/ml (E_{405} = 0.25) (Table 2), when the assay was performed by spectrophotometry at 405 nm. In comparative tests, the lowest concentration for the detection of BMoV in identical samples by infectivity tests was 100 ng/ml (Table 2). This result indicates that ELISA test is more sensitive than the infectivity test.

For the detection of BMoV in individual whole seeds or seeds in which the seed coat had been removed, ELISA tests were compared with infectivity tests to Top Crop bean plants. Estimates of the percentage for the detection of the virus in batches of seeds from BMoV-infected plants of 4 varieties were very similar for the ELISA and infectivity tests (Table 3). By both ELISA and infectivity tests BMoV was easily detected in whole seeds, although the percentage of the detection of virus in seeds in

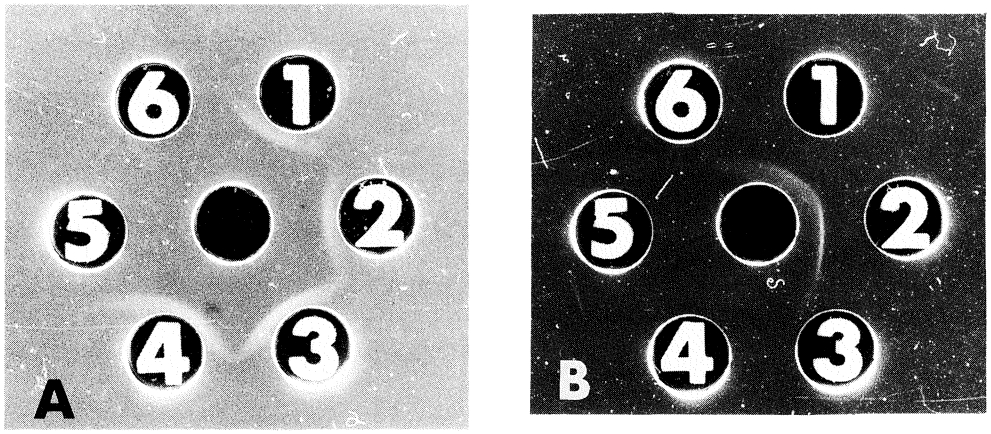

Fig. 3. Serological relationships. (A): Serological relationships between Indonesian and Thai blackgram mottle virus (BMoV). Center well: antiserum against Indonesian BMoV. Wells 1 and 2: purified Indonesian BMoV. Wells 3 and 4: purified Thai BMoV. Well 5: Sap from healthy bean leaf. Well 6: buffer. (B): Serological reaction of Indonesian BMoV to antisera against Indian BMoV and some other beetle-borne viruses. Center well: purified Indonesian BMoV. Wells 1, 2, 3, 4, 5, 6 are antisera against Indonesian BMoV, Indian BMoV, radish mosaic virus, southern bean mosaic virus, squash mosaic virus and turnip yellow mosaic virus, respectively.

Table 2. Sensitivity of enzyme-linked immunosorbent assay (ELISA) and infectivity tests for detection of purified blackgram mottle virus

	ELISA test	Infectivity test[a)]
Virus concentration (ng/ml)	Extinction value at 405 nm	Number of local lesions per leaf
10,000	3,20	85
5,000	3.18	47
2,500	3.17	21
1,000	3.06	11
500	2.40	3
250	1.27	2
100	0.48	1
50	0.25	0
25	0.11 (negative)	0
10	0.07 (negative)	0

a) Infectivity test was assessed by mechanical inoculation to primary leaves of the bean cultivar Top Crop.

which seed coats had been removed was very low (Table 3). It is suggested that the high positive reactions of whole seeds may be associated with the presence of seed coats. Therefore, the distribution of BMoV in seeds of the mungbean cultivar M7A was determined by ELISA tests. Mature seeds of M7A were treated with 10% trisodium phosphate for 10 min to inactivate the virus on the outer surface of seeds. Treated and untreated seeds were extracted for ELISA. For comparison, in mature seeds the seed coats and cotyledons with embryo were separated before extraction. Treatment of seeds with 10% sodium phosphate for 10 min had no significant effects on the absorbance values at 405 nm (Table 4). The absorbance values for the seed

Table 4. Distribution of blackgram mottle virus in whole seeds and in seed part of mature M7A mungbean cultivar

Seed part	Number of seeds tested	Extinction value at 405 nm
Treated whole seed[a)]	12	2.99
Untreated whole seed	12	3.51
Seed coat	12	3.19
Cotyledon with embryo	10	0.11

a) Mature seeds of M7A mungbean cultivar were treated with 10% trisodium phosphate for 10 min.

coats were markedly higher than those of the cotyledon with embryo (Table 4).

4. Discussion

Results on host range, symptomatology, transmission, morphology of virus particles and serological relationships indicated that the BMoV isolated from mungbean plants in Indonesia was closely related with the Indian and Thai BMoV. Antiserum against the Indonesian BMoV reacted identically with the Indonesian and Thai BMoV and also with the Indian BMoV. Indonesian BMoV reacted with antisera against the Indonesian and Indian BMoV, but did not react with antisera against other beetle-borne viruses. The present studies suggest that BMoV in Indonesia, Thailand and India may be the same virus.

The virus was experimentally transmitted by the bean leaf beetle, *Colposcelis signata* and golden green minute leaf beetle, *Balisepta fulvipes* collected from white clover and mugwort plants, respectively, in Japan. Further studies on beetle transmission using beetles collected in Indonesia are still needed for etiological studies on BMoV in the field in Indonesia.

One of the important purposes of this work was to develop rapid and highly sensitive methods for the detection of BMoV in mungbean seeds. Such methods would have to be relatively simple and inexpensive for practical screening of mungbean seeds. ELISA has been successfully used for the detection of the virus in seeds (2, 5, 6). Our results using purified BMoV indicated that the virus could be detected at concentrations as low as 50 ng/ml by ELISA test. ELISA was more sensitive than the infectivity test.

Our study of BMoV detection in immature mungbean seeds showed that the virus

was mainly located at high concentrations in the seed coat tissues but not on the outer surface of seeds. It seems that BMoV in the seed coats is rapidly inactivated during the maturation of seeds because seed transmission was very low (Table 3). It is suggested that the presence of BMoV in seeds in which seed coats had been removed is associated with the presence of the embryo or cotyledon. BMoV in the embryo or cotyledon may be correlated with seed transmission.

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