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Isolation and Characterization of a Geminivirus Causing Yellow Mosaic Disease of Horsegram (*Macrotyloma uniflorum* [Lam.] Verdc.) in India*

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With 2 figures

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Abstract

Horsegram yellow mosaic disease was shown to be caused by a geminivirus; horsegram yellow mosaic virus (HYMV). The virus could not be transmitted by mechanical sap inoculation. Leaf dip and purified virus preparations showed geminate virus particles measuring 15—18 × 30 nm. An antiserum for HYMV was produced and in enzyme-linked immunosorbent assay (ELISA) and immunosorbent electron microscopy (ISEM) tests HYMV was detected in leaf extracts of field-infected bambara groundnut, french bean, groundnut, limabean, mungbean, pigeonpea and soybean showing yellow mosaic symptoms. *Bemisia tabaci* fed on purified HYMV through a parafilm membrane transmitted the virus to all the hosts listed above but not to *Ageratum conyzoides*, okra, cassava, cowpea, *Croton bonplandianus*, *Lab-lab purpureus*, *Malvastrum coromandalianum* and tomato. No reaction was obtained in ELISA and ISEM tests between HYMV antibodies and extracts of plants diseased by whitefly-transmitted agents in India such as *A. conyzoides* yellow mosaic, okra yellow vein mosaic, *C. bonplandianus*, yellow vein mosaic, *M. coromandalianum* yellow vein mosaic, tomato leaf curl and cassava mosaic. HYMV was also not found to be related serologically to bean golden mosaic virus.

Zusammenfassung

Isolierung und Charakterisierung eines Zwillingsvirus, Ursache der gelben Mosaik-Krankheit bei „Horsegram" (*Macrotyloma uniflorum* [Lam.] Verdc.) in Indien

Horsegram yellow mosaic virus (HYMV) konnte als Ursache der horsegram yellow mosaic-Krankheit nachgewiesen werden. Durch eine mechanische Inokulation mit Pflanzensaft konnte der Virus nicht übertragen werden. Blattspitze- und gereinigte Viruspräparate wiesen Zwillingsviruspar-

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tikeln auf, die eine Größe 15—18 × 30 nm hatten. Ein HYMV-Antiserum wurde erzeugt und hiermit konnte in enzymgebundenen Immunosorbtionsuntersuchungen (enzyme-linked immunosorbent assay, ELISA) und Immunosorptionselektronenmikroskopieanalysen (immunosorbent electron microscopy, ISEM) HYMV in Blattextrakten von feldinfizierten Bambara-Erdnuß, grünen Bohnen, Erdnuß, Limabohnen, Mungobohnen, Pigeonerbsen und Sojabohnen mit gelben Mosaiksymptomen nachgewiesen werden. *Bemisia tabaci* mit gereinigten HYMV durch eine Parafilmmembran gefüttert, war in der Lage den Virus in alle die oben erwähnten Wirtspflanzen zu übertragen, aber nicht in *Ageratum conyzoides*, Okra, Maniok, Kuherbsen, *Croton sparsiflorus*, *Lab-lab purpureus*, *Malvastrum coromandalianum* und Tomaten. Keine Reaktion wurde in ELISA und ISEM-Untersuchungen zwischen HYMV-Antikörpern und Extrakten aus Pflanzen, die in Indien durch Weißfliege übertragene Viren wie *A. conyzoides* yellow mosaic, Okra yellow vein mosaic, *C. sparsiflorus* yellow vein mosaic, *M. coromandalianum* yellow vein mosaic, Tomatenblattkräusel- und Maniok-Mosaik befallen waren, ermittelt. Es wurde außerdem noch festgestellt, daß HYMV nicht mit bean golden mosaic virus serologisch verwandt ist.

Yellow mosaic disease of horsegram (*Macrotyloma uniflorum* [Lam.] Verdc.) (previously *Dolichos biflorus* Linn.) is one of the most important factors limiting yields of horsegram in south India (WILLIAMS *et al.* 1968, MUNIYAPPA *et al.* 1975, 1978). Incidence of the disease ranges from 50 to 100 % in both summer- and early rainy-season crops (MUNIYAPPA *et al.* 1978). Horsegram yellow mosaic disease has been shown to be transmitted by the whitefly *Bemisia tabaci* (Gennadius) (MUNIYAPPA and REDDY 1976). Yellow mosaic diseases have been reported on several legumes in India including french bean (*Phaseolus vulgaris* L.), (MARAMOROSCH and MUNIYAPPA 1981), groundnut (*Arachis hypogaea* L.) (SUDHAKAR RAO *et al.* 1980); limabean (*Phaseolus lunatus* L.) (CAPOOR and VARMA 1948); mungbean (*Vigna radiata* [L.] Wilczek) (NARIANI 1960, NENE 1972); pigeonpea (*Cajanus cajan* [L.] Mill sp.) (WILLIAMS *et al.* 1968, NENE 1972) and soybean (*Glycine max* [L.] Merr.) (SINGH *et al.* 1971). The yellow mosaic diseases of these hosts were described on the basis of symptoms, host range and transmission studies. Their causal agents have so far not been fully identified. In this paper we report isolation, purification and electron microscopy of a virus causing yellow mosaic disease of horsegram in south India, and on its serological relationships with causal agents of other whitefly transmitted diseases in India.

Materials and Methods

Colonies of the whitefly, *B. tabaci*, were maintained on cotton, *Gossypium hirsutum* cv. 'Varalakshmi', which was found to be immune to yellow mosaic disease. Only adult whiteflies were used in transmission experiments.

Leaves with typical yellow mosaic symptoms were collected from naturally infected horsegram plants (Fig. 1) and virus-free whiteflies were allowed to feed on them for 1 day before being transferred to healthy horsegram seedling in a screenhouse. After an inoculation access period of 1 day the whiteflies were killed by spraying 1 % dimethoate (30 EC). After typical yellow mosaic symptoms appeared on the inoculated plants, virus-free whiteflies were allowed 1 day acquisition access period. Exposed whiteflies were transferred to healthy french bean (*Phaseolus vulgaris* cv. 'Topcrop') seedlings. Subsequently virus was maintained in 'Topcrop' plants employing the same procedure.

First or second trifoliates of 'Topcrop' plants showing typical yellow mosaic symptoms were harvested 2 weeks after inoculation with whiteflies. They were quickly frozen with liquid nitrogen, crushed into fine powder, and homogenized in a blender with 0.1 M phosphate buffer, pH 8.0, containing 1 % 2-mercaptoethanol, 10 mM sodium ethylene diaminetetraacetate (EDTA) and 1 mM

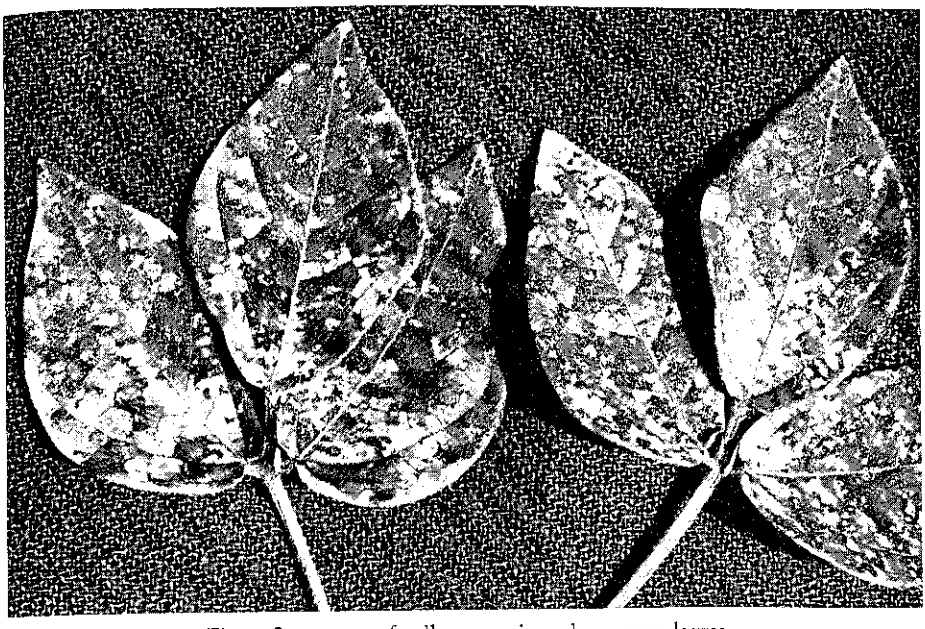

Fig. 1. Symptoms of yellow mosaic on horsegram leaves

cysteine hydrochloride; 3—4 ml buffer was used per g tissue (GOODMAN *et al.* 1977). The extract was squeezed through cheesecloth, clarified at 8,000 g for 20 min and polyethylene glycol (PEG) and sodium chloride were added to final concentrations of 4% and 0.2 M respectively. The mixture was incubated at 4°C for 3 h before centrifugation at 8,000 g for 20 min. The pellets were resuspended in 0.02 M phosphate buffer, pH 7.5 (PB) of which 0.5 ml was used per g of tissue. The suspension was clarified at 8,000 g for 10 min and treated with Triton X-100 in a concentration of 0.5%. Aliquots of 25 ml of the extract were layered on a 13 ml foot of 20% sucroce prepared in PB. Following centrifugation at 25,000 rpm for 3 h in a Beckman SW 27 rotor, the pellets were resuspended in 8.0 ml PB and subjected to rate zonal density centrifugation. The gradient columns were prepared by layering, respectively, 6, 9, 9 and 9 ml of 100, 200, 300 and 400 g/l of sucrose in PB and stored overnight at 4°C before use. Five ml of the virus preparation was layered on each gradient column and centrifuged at 25,000 rpm for 3 h in an SW 27 rotor. The two distinct light-scattering zones observed at 4.2 to 5.0 cm and 5.7 to 6.2 cm distance from the bottom of the tube were removed. Virus in purified preparations and leaf dips were negatively stained with 2% aqueous uranyl acetate, pH 3.7, or with 2% phosphotungstate (PTA), pH 7.0. Prior to staining some samples were also fixed for 30 min in 1.5% glutaraldehyde. All samples were examined in a Philips 201 C electron microscope. Particle measurements were made on micrographs magnified to 171,000 ×. The microscope was calibrated employing a germanium shadowed carbon replica having 21,600 lines/cm.

Virus preparations following one cycle of centrifugation in sucrose gradients was concentrated by pelleting at 35,000 rpm for 3 h. Approximately 1 mg virus (assuming an extinction coefficient of 7.0) resuspended in PB containing 5% sucrose was placed in a tube, covered with stretched parafilm (American Can Company, Marathon, Wisconsin). Unexposed *B. tabaci* adults, enclosed in another tube, were fed on purified virus through the membrane. After 1 day of acquisition 5—10 *B. tabaci* adults were released onto 7-day-old 'Topcrop' seedlings and were given an inoculation access period of 1 day. Exposed plants were sprayed with 1% dimethoate (30 EC).

Yellow mosaic infected groundnut, horsegram and 'Topcrop' leaves showing early symptoms were triturated in 0.1 M phosphate buffer, pH 8.0, containing 1% 2-mercaptoethanol. Extracts were inoculated onto 50—100 1-week-old 'Topcrop', groundnut, horsegram, lima bean, and soybean seedlings which were kept in dark for 2 days prior to inoculation. One set of inoculated plants was

kept in a screen house with temperatures ranging from 25—35 °C and another set was kept at 32 °C in a growth chamber in which 12 h of light (at an intensity of 1,500—2,000 lux) was alternated with 12 h of darkness.

New Zealand White inbred rabbits were given 3 intramuscular injections, each with 1 mg purified virus emsulfied with an equal volume of Freunds' incomplete adjuvant, at 1 week intervals. A single intravenous injection was given in the 4th week. Serum was collected at intervals from 2 weeks after the last intravenous injection. Antiserum titre was determined by precipitin ring test (REDDY and BLACK 1966). Serological relationships were determined by enzyme linked immunosorbent assay (ELISA) and immunosorbent electron microscopy (ISEM). The ELISA procedure was similar to that described by LISTER (1978) and RAJESHWARI *et al.* (1983). Coating globulins and conjugated globulins were used at concentrations of 10 µg/ml and 20 µg/ml, respectively. Formvar and carbon coated grids were floated on a 10 µl drop of 5 µg/ml protein-A (Sigma Chemicals) for 5 min and washed with 40 drops of 0.1 M phosphate buffer, pH 7.0 (0.1 M PB). Excess buffer was drained off with the edge of a filter paper and the grids were floated for 5 min on 10 µl d antiserum diluted to 1 : 100. The grids were washed with 0.1 M PB, floated for 15 min either on 20 µl d crude healthy or infected leaf extracts diluted to 1 : 10, subsequently grids were washed with 40 drops of distilled water. Staining was done with 2 % aqueous uranyl acetate, pH 3.7. The grids were viewed under a Philips 201-C electron microscope and particle counts made on 20 viewing fields at an instrument magnification of 45,000 ×.

Results

Purification

In preliminary experiments french bean cv. 'Topcrop', horsegram cv. 'H-H-2', lima bean, mungbean cv. 'PIMS-3', pigeonpea and soybean cv. 'KHSb-2' were tested for extracting the virus for purification. Concentration of virus was measured by spectrophotometric absorption of virus zones following rate-zonal centrifugation. Virus particles resembling geminiviruses were present in the lower zone, located at a depth of 4.2 to 5.0 cm from the bottom of the tube. 'Topcrop' tissue consistently yielded good virus preparations. Yields of 1—2 mg from 100 g tissue, assuming an extinction coefficient of 7.0 were obtained from 'Topcrop' tissue harvested 10—15 days after inoculation. Virus particles were disrupted when clarified extracts were treated with 10 % chloroform (v/v). However, treatment of extracts with 0.5 % Triton X-100 before layering on sucrose cushion yielded virus preparations with less host contaminants than untreated or 0.1 % Triton X-100 treated extracts.

Electron microscopy

Leaf dip and purified virus preparations showed virus particles of 15 to 18 × 30 nm size resembling geminiviruses (Fig. 2). Phytoferritin particles were also present in the virus zone. Uranyl acetate was more suitable than PTA for staining particles.

Infectivity tests

Whiteflies fed on purified virus preparations, acquired and transmitted the virus to 4 out of 48 'Topcrop' bean and 7 out of 50 horsegram seedlings used. Furthermore, whiteflies exposed to these infected plants transmitted the disease to healthy 'Topcrop' seedlings. In addition, all the infected plants gave positive

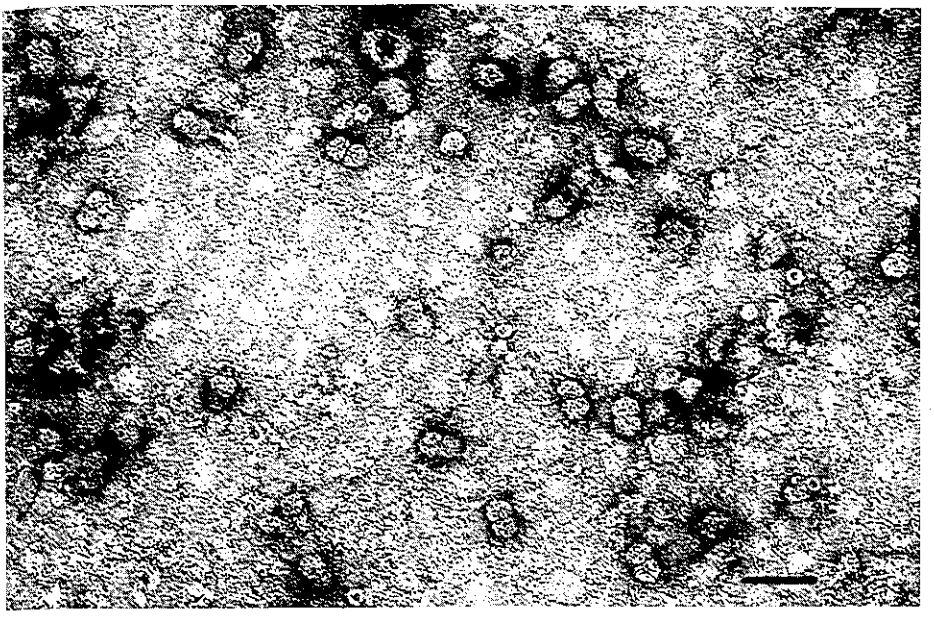

Fig. 2. Negatively stained virus particles purified from 'Topcrop' leaves. Bar represents 60 nm

reactions in ELISA. Eighty plants exposed to nonviruliferous whiteflies did not develop yellow mosaic symptoms nor did they contain viral antigen when tested by ELISA.

Mechanical transmission

The virus could not be mechanically transmitted with sap from infected 'Topcrop' bean, groundnut, and horsegram plants to 'Topcrop' bean, groundnut, lima bean, soybean and horsegram test plants.

Serological relationships

The titre of the antiserum was 1/80. In ELISA tests, extracts from leaves of bambara groundnut (*Voandzeia subterranea*), french bean (*Phaseolus vulgaris* Local), groundnut (*Arachis hypogaea*), horsegram (*Macrotyloma uniflorum*), limabean (*Phaseolus lunatus*), mungbean (*Vigna radiata*), pigeonpea (*Cajanus cajan*), and soybean (*Glycine max*), naturally infected with yellow mosaic cross reacted strongly with the HYMV antiserum. In addition, a relatively large number of particles were trapped from these extracts by ISEM (Table). In ELISA, extracts of leaves infected with yellow vein mosaic of *A. conyzoides, A. esculentus, C. bonplandianus, M. coromandalianum,* leaf curl of tomato and cassava mosaic virus gave absorption values at A 405 similar to healthy leaf extracts. In addition the number of particles trapped by HYMV antiserum in ISEM were comparable to those trapped with normal serum (Table). In ELISA and ISEM tests extracts of yellow mosaic infected french bean and horsegram failed to react with bean golden mosaic virus (BGMV) antiserum obtained from Dr. R. M. GOODMAN.

Table
Serological relationships of HYMV with other virus causing whitefly transmitted diseases in India

Antigen tested	Absorbance at 405 nm		No. of gemini-virus particles trapped by HYMV antiserum*	No. of gemini-virus particles trapped by normal serum*
	Healthy leaf extract (1 : 10)	Infected leaf extract (1 : 10)		
French bean yellow mosaic	0.009	0.428	410	2
Groundnut yellow mosaic	0.008	0.215	268	8
Horsegram yellow mosaic	0.006	0.436	360	12
Lima bean yellow mosaic	0.004	0.384	386	7
Mungbean yellow mosaic	0.001	0.368	284	11
Soybean yellow mosaic	0.001	0.392	295	20
Bambara groundnut yellow mosaic	0.006	0.261	259	5
Ageratum conyzoides yellow vein mosaic	0.008	0.012	—	—
Okra yellow vein mosaic	0.020	0.001	—	—
Croton bonplandianus yellow vein mosaic	0.018	0.022	—	—
Malvastrum coromandalianum yellow vein mosaic	0.005	0.005	—	—
Tomato leaf curl	0.004	0.005	—	—
Cassava mosaic	0.001	0.004	18	17
Cowpea mild mottle infected soybean	0.006	0.009	—	—

* Counts represent values from 20 random viewed fields at a magnification of × 45,000; — no particles could be trapped.

Discussion

Horsegram yellow mosaic virus was identified as a geminivirus on the basis of particle morphology. Geminiviruses have earlier been shown to cause yellow mosaic diseases transmitted by whiteflies (GOODMAN 1981, GOODMAN *et al.* 1977, HONDA *et al.* 1983, KIM *et al.* 1978). The symptoms produced in mungbean by HYMV are similar to those of mungbean yellow mosaic (MYMV) reported from Thailand. However, the MYMV was mechanically transmissible (HONDA *et al.* 1983) and employing the same conditions HYMV could not be transmitted mechanically. Antiserum of the yellow mosaic virus reported from Thailand has not been produced (HONDA, per. comm.) for comparative studies. Based on serology we regard HYMV as distinct from BGMV though both the geminiviruses cause very similar symptoms on 'Topcrop' bean.

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