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Natural Occurrence of a Strain of Cowpea Mild Mottle Virus on Groundnut (*Arachis hypogaea*) in India*)

By

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With 6 figures

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Abstract

Vein-clearing followed by downward rolling and necrosis of leaves and severe stunting of groundnut (*Arachis hypogaea*) plants were caused by cowpea mild mottle virus (CMMV). The virus was readily transmitted by mechanical sap inoculations to groundnut and to 10 plant species belonging to Leguminosae, Chenopodiaceae and Solanaceae. *Chenopodium quinoa* and *Beta vulgaris* were good diagnostic hosts. Diseased sap remained infective at 10^{-3} but not 10^{-4}, when stored 8 to 9 days at 25 °C; for 10 min at 75 °C but not 80 °C. In limited tests, virus was not seed-transmitted in groundnut or soybean. Virus was transmitted by *Bemisia tabaci* but not by *Aphis craccivora* or *Myzus persicae*. An antiserum for CMMV was produced and virus was serologically related to CMMV reported on cowpea and groundnut crinkle virus (GCV) from West Africa. Employing carbon diffraction grating replica as a standard the modal length of virus particles was found to be 610 nm. Infected cells contained large number of virus particles associated with endoplasmic reticulum.

Zusammenfassung

Das natürliche Vorkommen eines Stammes des Cowpea mild mottle Virus auf Erdnüssen (*Arachis hypogaea*) in Indien

Das Cowpea mild mottle-Virus (CMMV) ruft bei Erdnußpflanzen (*Arachis hypogaea*) eine Adernaufhellung gefolgt von einem nach unten gerichteten Einrollen und einer Nekrose der Blätter sowie eine schwere Verkümmerung der Pflanze hervor. Das Virus wird leicht

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durch eine mechanische Saftinokulation in Erdnüsse und in zehn Pflanzenarten, die zu den Leguminosae, Chenopodiaceae und Solanaceae gehören, übertragen. *Chenopodium quinoa* und *Beta vulgaris* waren für die Diagnose gute Wirte. Infizierter Saft blieb bei einem Verdünnungsendpunkt von 10^{-3} aber nicht bei 10^{-4} infektiös, wenn er 8 bis 9 Tage bei 25 °C gelagert wurde, der thermale Inaktivierungspunkt betrug bei 10 min 75 °C, nicht jedoch 80 °C. In begrenzten Tests war das Virus nicht samenübertragbar bei Erdnuß oder Sojabohne. Das Virus wurde durch *Bemisia tabaci* aber nicht durch *Aphis craccivora* oder *Myzus persicae* übertragen. Es wurde ein Antiserum für CMMV produziert, und das Virus war serologisch verwandt mit CMMV, von dem auf Augenbohnen berichtet wurde, und mit Groundnut crinkle-Virus (GCV) aus West-Afrika. Mit Hilfe des Kohleabdrucks eines Beugungsgitters als Standard wurde die normale Länge der Viruspartikel mit 610 nm festgestellt. Infizierte Zellen enthielten eine große Anzahl von Viruspartikeln, die sich mit dem endoplasmatischen Reticulum verbunden hatten.

A disease of groundnut (*Arachis hypogaea*) characterised by severe stunting, downward rolling and necrosis of leaves was observed in farmers' crops in Maharashtra, Punjab, Uttar Pradesh, and Tamil Nadu states of India. Although the disease was widely distributed, the incidence was usually less than one per cent. It was suspected that the disease was caused by a virus but the symptoms did not agree with those of any groundnut virus disease previously described in India. These include peanut mottle virus (PMV) (REDDY *et al.* 1978), bud necrosis caused by tomato spotted wilt virus (TSWV) (GHANEKAR *et al.* 1979) and peanut green mosaic virus (PGMV) (SREENIVASULU *et al.* 1981). In this paper we report on the occurrence, isolation, and identification of a strain of cowpea mild mottle virus (CMMV) in India.

Materials and Methods

The disease was established in a screenhouse by graft infecting healthy TMV-2 groundnut cultivar. Then symptomatic leaves were extracted and mechanically inoculated onto groundnut and *Chenopodium quinoa* where five single lesion transfers were made. Single lesion isolates were maintained in groundnut plants; all plant extracts were prepared in 0.05 M phosphate buffer, pH 7.0, containing 0.02 M 2-mercaptoethanol (PBM).

A few plant species produced chlorotic or necrotic lesions or both on mechanically inoculated leaves. However, *C. quinoa* consistently produced the greatest number of chlorotic lesions and was selected as an assay host. All assays were performed on fully expanded leaves, employing at least eight leaves for each test.

Five or more plants of each species were inoculated and maintained in a screenhouse for about 2 months. Both inoculated and non-inoculated leaves were assayed on *C. quinoa*.

The physical properties of the virus were determined on crude groundnut leaf extracts diluted to 10^{-1} in PBM. For determining thermal inactivation point, a ml of sap was heated for 10 min in a water bath at various temperatures.

Aphis craccivora and *Myzus persicae* maintained on healthy groundnut and cabbage cv. Local plants respectively, were starved for 1 hour before they were allowed acquisition feeds of 2 to 10 min or 1 to 2 hours on young infected groundnut leaves and a day long inoculation feed on healthy groundnut plants. In another experiment insects were allowed acquisition feeds of 1 day followed by 4 days of inoculation feeds. Over 200 aphids in each experiment were given acquisition feeds and groups of 20 individuals were transferred to each healthy groundnut plant.

Adults of *Bemisia tabaci* were maintained on *Gossypium hirsutum* cv. Varalakshmi and tested on groundnut and soybean (*Glycine max*, cv. Bragg). Acquisition feeds consisted

of 1, 3 and 24 hours on infected groundnut and soybean plants. Then exposed insects were released (five per plant) on healthy groundnut and soybean plants for a day.

At the end of the inoculation feeds all insects were sprayed with 0.025 % Metasystox (demetonmethyl, Bayor, India). Test plants were maintained in a screenhouse for observation.

Transmission tests were done on seeds collected from infected groundnut and soybean plants. Seedlings were raised in sterile soil in a screenhouse.

The details of the method employed for purification are described by RAJESHWARI *et al.* (in preparation). Antiserum was produced in a rabbit following four intramuscular injections at weekly intervals with a mg of purified virus emulsified with an equal volume of Freund's incomplete adjuvant. Serum was collected 2 weeks after the last injection and titred by the precipitin ring test employing purified virus of 250 μg/ml. Serological relationships were tested by enzyme-linked immunosorbent assay (ELISA). Leaf extracts for ELISA were prepared in PBS (0.02 M phosphate buffer, 0.15 M sodium chloride, 0.003 M potassium chloride, pH 7.4) containing 0.05 % Tween-20 and 2 % polyvinylpyrrolidone, of mol. wt. 40,000. All dilutions from leaf tissue were based on the original weight and purified virus was diluted on the assumption of an extinction coefficient of 3.0.

The procedure described previously by CLARK and ADAMS (1977) and modified by LISTER (1978) was employed. The γ-globulins were extracted with 18 % sodium sulphate and adjusted to 1 mg/ml (A 280 nm =1.4) and then conjugated with alkaline phosphatase (Type VII, Sigma Chemical Co.) at an enzyme globulin ratio of 2 : 1 in the presence of 0.06 % glutaraldehyde. Wells in polystyrene micro ELISA plates (Dynatech Laboratories) were filled with 0.2 ml of reagents. Coating globulin was used at 2.0 and 4.0 μg/ml, respectively, for homologous antiserum and CMMV antiserum provided by Dr. A. A. BRUNT, Glasshouse Crops Research Institute, Littlehampton. Coating globulin at 10 μg/ml was used from groundnut crinkle virus (GCV) antiserum provided by Dr. C. FAUQUET, ORSTOM, Ivory Coast. In all tests conjugated globulins of homologous antiserum at 1/400 dilution was used. Incubation with substrate was at room temperature for 30 min to 1 hour and the reaction was stopped by adding 0.05 ml of 3 M sodium hydroxide to each well. Assay was by reading absorbance (A 405 nm) in a Gilford 250 spectrophotometer. All the tests were done in duplicate and the results were reproducible.

Infected leaves of groundnut and soybean were cut into small pieces and fixed in 3 % glutaraldehyde and 0.1 M phosphate buffer, pH 7.3, for 1 hour. Fixed tissues were rinsed in phosphate buffer containing 0.5 % sucrose, washed in cold phosphate buffer for 3 hours, post-fixed in 2 % osmium tetroxide for 3 hours at room temperature and then washed with distilled water. Specimens were dehydrated in graded series of acetone and embedded in Epon 812. Ultrathin sections were cut with glass knives, stained with uranyl acetate, and lead citrate (VENABLE and COGGESHALL 1965) and examined in a Philips model 201 C electron microscope.

Also freshly harvested leaflets with vein-clearing symptoms, were cut into small strips. Freshly cut surface of several leaflets were quickly dipped in distilled water droplets and held until the droplet turned light green, then it was transferred to 300 mesh copper grids coated with formvar membrane and carbon. After 5 min excess liquid was removed with filter paper, stained with 2 % neutral phosphotungstic acid, and examined. Pelleted virus from rate zonal centrifugation was negatively stained. Particle measurements were made on micrographs taken at × 20,000. The microscope was calibrated employing germanium shadowed carbon replica having 21,600 lines/cm. The actual magnification was used for computing the size of virus particles.

Results

Symptoms in groundnut

Ten to 15 days after sap inoculations, plants showed vein-clearing and downward rolling of newly emerged quadrifoliate leaves (Fig. 1). Later tissue necrosis of leaves and petioles occurred and leaves dropped off.

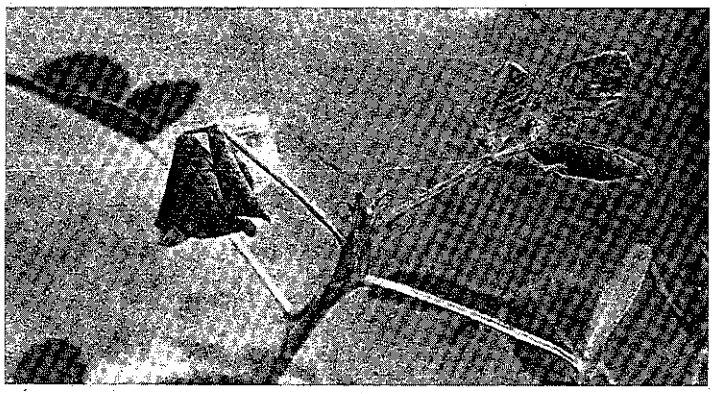

Fig. 1. Typical downward curling and vein-banding symptoms induced by CMMV

Host range

Beta vulgaris, Cajanus cajan cv. Sharda, *Chenopodium amaranticolor,* and *Cyamopsis tetragonoloba* produced only local lesions. *Chenopodium quinoa* produced chlorotic lesions on inoculated (Fig. 2) and newly produced leaves.

Glycine max cv. Bragg produced necrotic lesions and veinal necrosis on inoculated leaves. Systemically severe mosaic and apical necrosis were observed (Fig. 3). The virus induced systemic mosaic symptoms on *Canavalia ensi-*

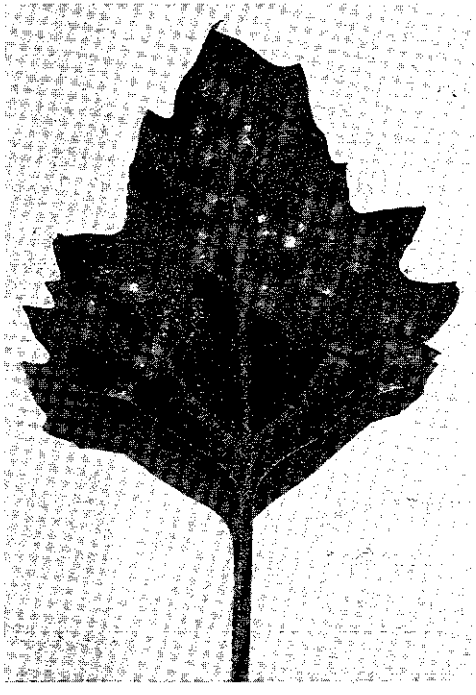

Fig. 2. Chlorotic local lesions on *C. quinoa* induced by CMMV

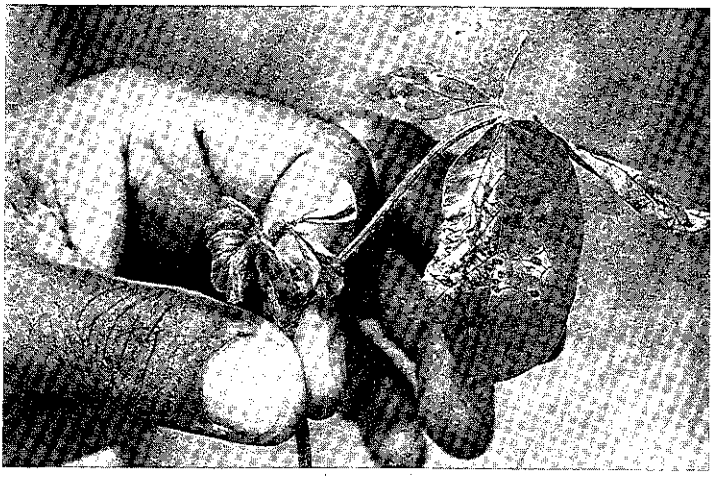

Fig. 3. Necrosis on inoculated leaves and systemic curling symptoms induced by CMMV on soybean

formis, Cassia occidentalis, Nicotiana clevelandii, Phaseolus vulgaris cvs. Kentucky Wonder and Kintoki, *Pisum sativum* cv. Bonneville and *Vigna unguiculata* cv. Early Ramshorn while *V. unguiculata* cv. C-152 was infected symptomlessly.

The virus failed to infect the following plants: *Cassia obtusifolia, Crotalaria juncea, Cucumis sativus* cv. National Pickling, *Datura stramonium, Lycopersicon esculentum* cv. Pusa Ruby, *Nicotiana benthamiana, N. clevelandii* × *N. glutinosa, N. glutinosa, N. rustica, N. tabacum* cv. Turkish, *Petunia hybrida, Phaseolus vulgaris* cv. Topcrop, *Vigna mungo, V. radiata* and *Zinnia elegans.*

Transmission

The virus was readily transmitted by sap inoculation. *A. craccivora* and *M. persicae* failed to transmit the virus. Results show (Table) virus transmission by *B. tabaci.* Acquisition feeds of 1 hour or 24 hours had no marked effect upon viruliferousness; similar rates of transmission were found. Infected plants were confirmed by bio- and sero-assays.

Seed transmission

Infected groundnut plants produced few seeds, however, over 90% of 153 groundnut seed and 510 soybean seed germinated and none showed typical disease symptoms. Randomly selected seedlings were negative on *C. quinoa* assays, also.

Physical properties

The thermal inactivation point was between 75 °C and 80 °C and the dilution end point was between 10^{-3} and 10^{-4}. The virus remained infective at room temperature (25 °C to 30 °C) for 8 days but not 10 days.

Table
Transmission tests with *Bemisia tabaci* on cowpea mild mottle virus from groundnut and soybeans

Acquisition source	Acquisition access period (hours)	No. of plants that became infected over no. exposed*)	
		groundnut	soybean
Infected groundnut	1	6/15	11/14
Infected groundnut	3	6/16	10/16
Infected groundnut	24	14/26	15/19
Infected soybeans	1	5/13	12/14
Infected soybeans	3	8/22	15/21
Infected soybeans	24	10/32	29/36
Healthy groundnut	24	0/41	0/38
Healthy groundnut	24	0/50	0/58

*) Five insects were transferred to each of exposed plants and allowed 1 day of inoculation feeding period. Extracts from one infected plant from each exposed group were simultaneously assayed on *Chenopodium quinoa* and tested in ELISA employing cowpea mild mottle virus antiserum produced at ICRISAT.

Serology

The homologous antiserum was 1/500 in precipitin ring test. Results of ELISA show that infected groundnut and soybean extracts had a titre of 1/3200 and 1/12800, respectively, when tested with globulins from homo-

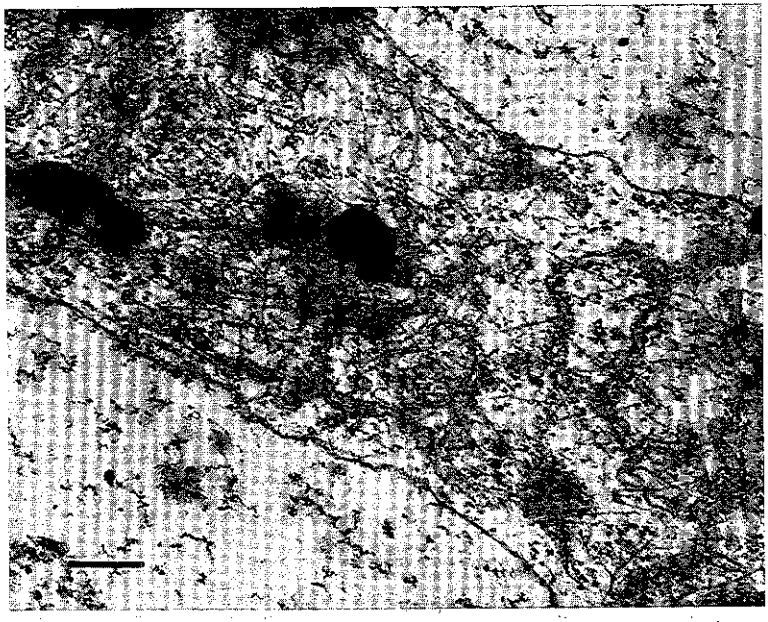

Fig. 4. Electron micrograph of a thin section of a leaf of CMMV-infected soybean showing virus particles in cytoplasm, associated with endoplasmic reticulum. Bar represents 300 nm

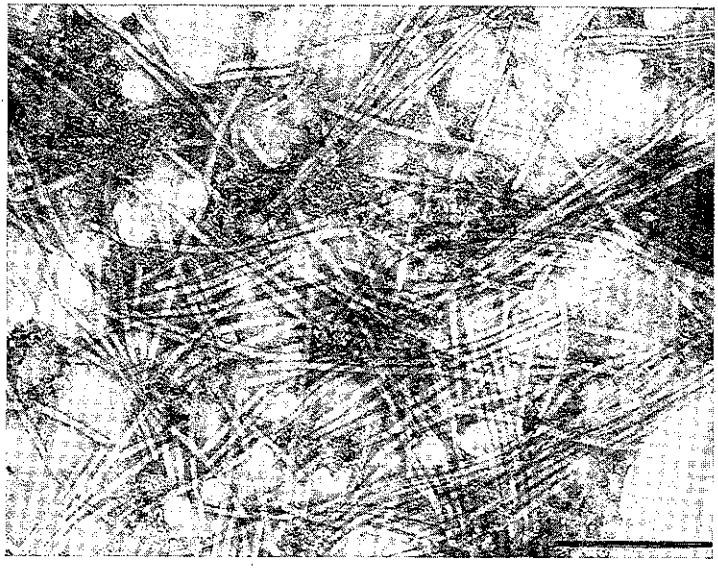

Fig. 5. Negatively stained CMMV particles purified from soybean leaves. Bar represents 300 nm

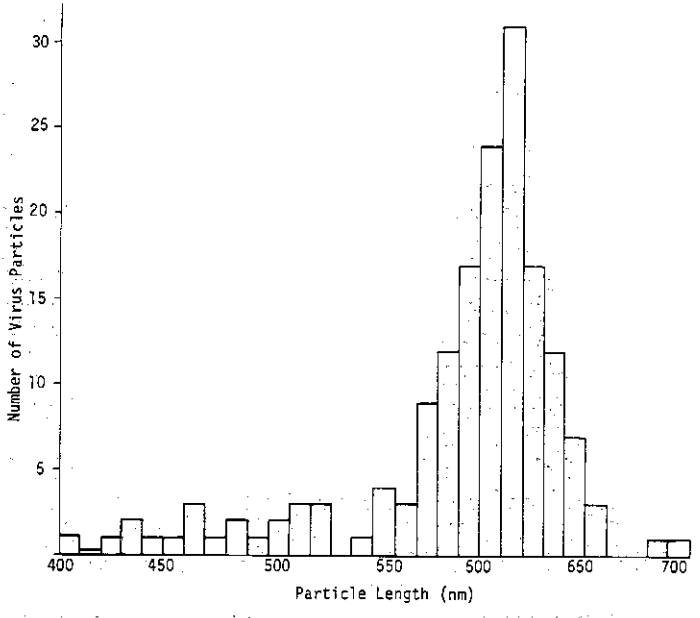

Fig. 6. Histogram of particle length distribution in crude soybean leaf extract

logous antiserum. CMMV antiserum from Dr. BRUNT gave titres of 1/800 and 1/3200, with infected groundnut and soybean extracts, respectively. Extracts from all healthy controls, peanut mottle virus infected and clump virus infected leaf extracts gave no reaction, with both homologous and Dr. BRUNT's

CMMV antiserum. Both antisera also reacted with purified virus to levels of about 50 ng/ml with homologous and 300 ng/ml with BRUNT's CMMV antiserum. GCV antiserum also reacted with infected groundnut and soybean extracts up to 1/400 and 1/1600, respectively, and with 500 ng/ml of purified virus.

Electron microscopy

In thin sections of infected tissue, scattered virus particles were present in cytoplasm, associated with endoplasmic reticulum (Fig. 4).

Leaf dip preparations from both peanut and soybean leaves and purified preparations showed slightly flexuous rods of 15 nm diameter (Fig. 5) with a modal length of 610 nm (Fig. 6) as determined on leaf dip preparations. Particles were aggregated in purified preparations.

Discussion

On the basis of serology, electron microscopy and host range reactions, the groundnut virus was identified as a strain of cowpea mild mottle virus (CMMV). Host range, symptoms produced on susceptible hosts were similar to those of CMMV (BRUNT and KENTEN 1973) and it was serologically related to CMMV from W. Africa. However, unlike the W. African CMMV isolate ours infected *Pisum sativum*, showed a higher thermal inactivation point and was not seed-transmitted.

GCV was earlier claimed to be a strain of carnation latent virus (CLV) and the serological relationships of GCV with CMMV was not investigated (DUBERN and DOLLET 1981). Symptoms of CMMV on groundnut markedly differs from GCV and host range of GCV is restricted to legumes. Our results show that CMMV is serologically related to GCV. CMMV was earlier shown to be distantly related to CLV (BRUNT and KENTEN 1973). Among other rod-shaped viruses, sweet potato mild mottle virus (SPMMV) from E. Africa (HOLLINGS *et al.* 1976) and cucumber vein yellowing virus (CVYV) from Israel (SELA *et al.* 1980) also were transmitted by whiteflies. Both SPMMV and CVYV differ from CMMV in host range, physical properties, and particle length. CVYV was shown to contain double-stranded DNA (SELA *et al.* 1980) and SPMMV, RNA (HOLLINGS *et al.* 1976).

On the basis of electron microscopy and polypeptide molecular weight, CMMV was grouped under carlaviruses (BRUNT and KENTEN 1974). Serological tests on CMMV and GCV (DUBERN and DOLLET 1981) provide additional proof that CMMV should be grouped under carlaviruses. Results of IWAKI *et al.* (1982) and our preliminary results of polypeptide and nucleic acid analyses of CMMV (RAJESHWARI *et al.*, in preparation) show that it resembles carlaviruses.

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