Properties of a Cucumber Mosaic Virus Strain Naturally Infecting Chrysanthemum in India

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ABSTRACT

Srivastava, K. M. , Raj, S. K., and Singh, B. P. 1992. Properties of a cucumber mosaic virus strain naturally infecting chrysanthemum in India. Plant Dis. 76:474-477.

Diffused chlorosis, chlorotic dots near veins, and stunting plants were observed in *Chrysanthemum* × *morifolium*. A virus isolate was purified and characterized as chrysanthemum strain of cucumber mosaic virus (CMV-CI), based on host reaction, in vitro properties, particle morphology (29 nm), molecular weight of coat protein subunit (24.5 kDa), nucleic acid (five RNA species), and serological relationships with other cucumoviruses.

Among cucumoviruses, chrysanthemum aspermy virus (CAV) has been frequently recorded on chrysanthemums (3,4,7,12,13,16). Chrysanthemum mild mottle virus (CMMV) reported on chrysanthemum in Japan is serologically related to tomato aspermy virus (TAV). TAV, CAV, and CMMV are closely related and are considered strains of one virus (5). Chrysanthemums are normally not infected by CMV-strains (9); therefore, the plant is considered a diagnostically useful host for differentiation between CMV and TAV or CAV. However, it had been recorded as a host of a CMV strain during experimental inoculation (15). During 1988–89 we observed disease symptoms on several varieties of chrysanthemum growing under natural conditions in the gardens of the National Botanical Research Institute in Lucknow, India. The symptoms appeared different from those caused by TAV or CAV. In this communication we report the biological and serological properties of this unusual strain of CMV naturally infecting chrysanthemums.

MATERIALS AND METHODS

Diseased *Chrysanthemum* × *morifolium* Ramat. plants growing under natural conditions in pots were brought to a greenhouse and served as the source of starting material. Infected leaves were macerated in EB (100 mM phosphate buffer, pH 7; 0.1% sodium sulfite; and 5 mM EDTA) in a ratio of 1:2 (w/v). Inoculum prepared in this way was rubbed on *Nicotiana tabacum* L. 'White Burley,' *Chenopodium album* L., and *C.* × *morifolium*. Lethal necrosis in White Burley tobacco, chlorotic lesions on *C. album*, and diffused chlorosis or chlorotic dots near veins were invariably induced by six independent inocula prepared from naturally infected *C.* × *morifolium*. To obtain pure culture of the virus, five serial, single-lesion transfers were made on *C. album*. Inoculum from the lesions produced after the fifth transfer was used for inoculation of *C.* × *morifolium* and White Burley tobacco. The lesion-purified culture on *C.* × *morifolium* (LPC) on White Burley tobacco (LPT) was used in subsequent studies unless otherwise stated.

Host range of the isolate was tested by inoculum prepared from infected leaves of *C.* × *morifolium* (LPC) in EB (1:3) on at least five plants of every species tested (Table 1). Back inoculations were made on *C. album* to ascertain the presence or absence of virus in inoculated plants.

The aphids *Myzus persicae* (Sulzer) and *Aphis gossypii* Glover were used for transmission tests, with White Burley tobacco as both donor and recipient host. The aphids, starved for 2 hr, were given an acquisition access of 2 min on infected leaves and an inoculation access of 2 hr on test plants. At least 10 aphids per plant were used for infection, and 10 plants were tested with each aphid species.

Properties of the virus in sap of *C.* × *morifolium* leaves, including dilution end point, thermal inactivation point, and longevity in vitro, were carried out as detailed (14), with *C. album* as local lesion host.

Purification of the virus was done from infected leaves of White Burley tobacco as described (10), with some modifications. Briefly, 100 g of infected tissue, harvested 4–5 days after inoculation, was ground in 3 vol of EB (w/v), squeezed through double folds of muslin cloth, and mixed with chilled chloroform and Triton X-100 so as to achieve a final concentration of 15 and 1%, respectively. Aqueous supernatant obtained after low-speed centrifugation (10,000 *g* for 10 min)

Table 1. Host range and symptoms induced by chrysanthemum strain of cucumber mosaic virus (CMV-CI), using lesion-purified inoculum maintained on *Chrysanthemum* × *morifolium*

Host range	Leaf symptoms[a]	
	Local	Systemic
Solanaceae		
Nicotiana rustica L.	NR	NAS
N. tabacum L. 'Samsun NN'	NR, NLP	M
N. tabacum L. 'White Burley'	NR, SVN	SPN, SVN, S, D
N. plumbaginifolia Viv.	NR	NAS
Solanum nigrum L.	CS	M
Capsicum annuum L.	CS	NAS
Chenopodiaceae		
Chenopodium amaranticolor Coste & A. Reynier	NLL	−*
C. murale L.	CLL	−*
C. album L.	CLL	−*
Spinacia oleracea L.	CLL, NLL	−*
Leguminosae		
Vicia faba L. 'Sutton'	NLL	−
Vigna unguiculata (L.) Walp.	−*	−
Lablab purpureus (L.) Sweet	−*	−
Phaseolus vulgaris L.	−*	−
Cucurbitaceae		
Cucumis sativus L. 'Long Green'	NLL, VC, M	NAS

[a] CLL = chlorotic local lesions; CS = chlorotic spots; D = death of plants; M = mosaic; NAS = no apparent symptoms, but presence of virus detected; NLP = necrotic line pattern; NLL = necrotic local lesions; NR = necrotic rings; S = stunt; SPN = severe petiole necrosis; SVN = severe veinal necrosis; VC = veinclearing; − = no symptoms (back inoculation not done); and −* = no symptoms (back inoculation test done on *C. album*, with negative results).

Accepted for publication 9 May 1991.

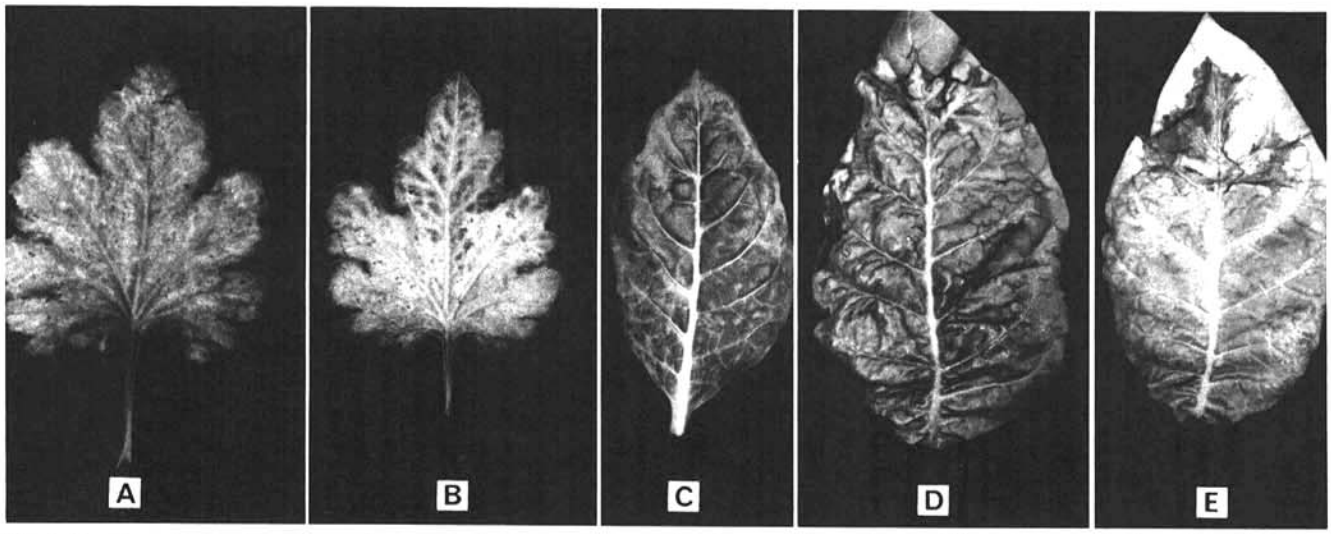

Fig. 1. Symptoms induced by chrysanthemum strain of cucumber mosaic virus on *Chrysanthemum* × *morifolium*, showing (**A**) mild diffused chlorosis; (**B**) severe chlorosis accompanied by necrosis; (**C**) systemically infected leaf of *Nicotiana tabacum* 'Samsun NN,' showing mosaic; and (**D,E**) inoculated leaves of *N. tabacum* 'White Burley,' showing veinal necrosis.

was centrifuged in a Beckman Ti 45 rotor (40,000 rpm, 2 hr) over a 15% sucrose pad prepared in EB. The pellet was suspended in 10 ml of buffer A (5 mM sodium borate, pH 9.0; 0.5 mM EDTA; and 1% Triton X-100) stirred overnight at 4 C, clarified by low-speed centrifugation (10,000 *g* for 10 min), and again centrifuged in a Beckman Type 50 rotor (40,000 rpm, 2 hr). The pellet was resuspended in buffer B (buffer A without Triton X-100). Further purification was done by centrifugation on a linear sucrose gradient (5–25%) in a Beckman SW-25 rotor (24,000 rpm, 3 hr), followed by removal of sucrose from the fraction containing the virus by overnight dialysis in 1 L of buffer B.

The yield of virus per kilogram of tissue was calculated by assuming the extinction coefficient at 260 nm to be 5 (6). Infectivity of purified preparation was tested on three plants each of *C.* × *morifolium, N. tabacum* cv. White Burley, and *C. album* by inoculating 10 mg/ml of virus preparation. Particle morphology and size was established by examination of stained, purified preparations of uranyl acetate (2%, pH 4.2) in a Philips CM 10 electron microscope. The molecular weight of viral coat protein subunit was determined by discontinuous SDS-PAGE in 10% gel following the method described (11).

Viral nucleic acid was isolated by disrupting the particles with 1% SDS and equal volume of phenol. Subsequent extractions were made by phenol + chloroform followed by chloroform alone. Nucleic acid was precipitated in a routine way from the aqueous phase (17). Nucleic acid preparations suspended in appropriate buffer were treated with DNase (50 μg/ml), RNase (20 μg/ml), or S_1 nuclease (50 μg/ml) separately (16) at 37 C for 1 hr, and nucleic acid was precipitated from them after treatment with phenol and suspended in 10 mM Tris-HCl, pH 7.5. For infectivity tests, the nucleic acid samples (200 μg/ml) obtained as detailed above were inoculated on White Burley plants.

Electrophoresis of nucleic acid preparation (5 ug/ml) was carried out on 1.2% agarose gel under nondenaturing conditions in TBE buffer (17). The gel was stained with 0.5 μg/ml ethidium bromide and observed under UV light.

To establish serological relationship, both Ouchterlony double-diffusion test (ODDT) and direct antigen coating enzyme-linked immunosorbent assay (DAC-ELISA) were carried out. Crude antigen for ODDT was prepared by triturating 1 g of young infected leaves in 2 ml of PB (100 mM phosphate buffer, pH 7.0) while purified antigen was diluted to final concentration of 5 mg/ml. The wells were charged with the antigen (crude or purified) and antiserum (one-fourth dilution) and incubated at room temperature (30–32 C) in a humid chamber. In DAC-ELISA, coating of wells was done with 10 μg/ml of purified virus (200 μl per well). The antisera to various isolates were used at a dilution of 1:10,000. Secondary antibody (goat anti-rabbit) conjugated to alkaline phosphatase was used at a dilution of 1:1,000.

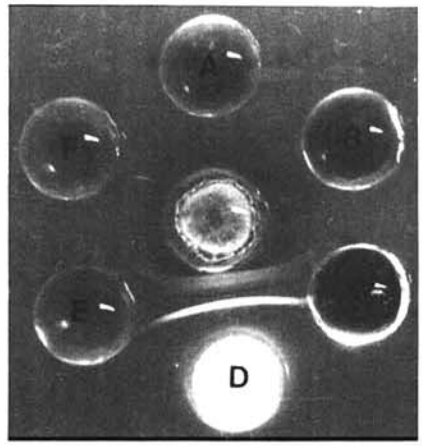

Fig. 2. Ouchterlony double-diffusion test showing negative reaction of chrysanthemum isolate and other cucumber mosaic virus (CMV) isolates with antiserum to chrysanthemum aspermy virus (CAV). Center well: antiserum to CAV (PVAS-24). Peripheral wells: (**A**) CMV from *Petunia* × *hybrida*, (**B**) CMV from *Physalis minima*, (**C**) sap from healthy *C.* × *morifolium*, (**D**) CAV-CI chrysanthemum isolate, (**E**) CMV-CI chrysanthemum isolate under investigation, and (**F**) sap from healthy White Burley tobacco.

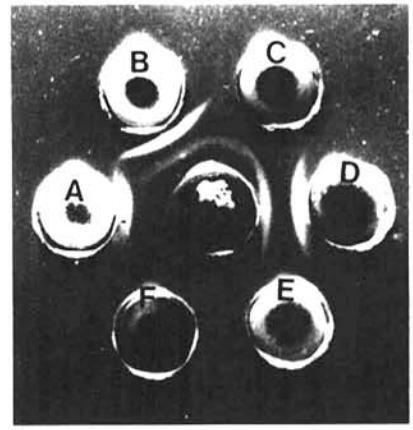

Fig. 3. Ouchterlony double-diffusion test showing positive reaction of chrysanthemum isolate and other cucumber mosaic virus (CMV) isolates with antiserum to CMV-T. Central well: antiserum to CMV-T. Peripheral wells: crude antigens (**A**) CMV-CI (chrysanthemum isolate), (**B**) CMV-Pet from *Petunia* × *hybrida*, (**C**) CMV-O from *Ocimum americanum*, (**D**) CMV from *Physalis minima*, (**E**) chrysanthemum aspermy virus (CAV) from chrysanthemum, and (**F**) sap from healthy White Burley tobacco.

The origins of CMV-C, CMV-S and CMV-D are as described (8,19). CMV-L is a strain from lupins described earlier (2). CAV (PVAS-24) was purchased from ATCC, and antisera to CMV-T and TAV-N were gifts from R. I. B. Francki of Australia.

RESULTS

Naturally infected *C.* × *morifolium* showed diffused chlorosis, chlorotic dots near veins, and necrosis of leaves (Fig. 1A and B). At later stages of infection, older leaves showed browning of major veins, leading to downward cupping and, finally, abscission. The virus was transmitted by mechanical means to several hosts (Table 1). *M. persicae* and *A. gossypii* transmitted the virus to an extent of 50 and 10%, respectively, when White Burley tobacco was used as both donor and recipient host.

The virus in sap of *C.* × *morifolium* retained infectivity after it was diluted to 10^{-3}, heated at 55 C for 10 min, and stored for 2 days at ambient temperature (28–30 C).

Gradient purified virus preparations produced symptoms on *C.* × *morifolium* identical to those observed in nature. The yield of the virus in tobacco leaves was 210 mg/kg of fresh tissue when harvested 5–6 days after virus inoculation. Purified virus gave a characteristic peak of nucleoprotein, with absorption maxima and minima at 260 and 240 nm, respectively. A ratio of A_{260}:A_{280} was calculated as 1.58. Spherical particles averaging 29 nm (based on measurement of 178 particles) were observed in preparations stained with uranyl acetate. Staining with neutral phosphotungstic acid (2%), however, caused disruption of particles. Molecular weight of viral coat protein subunits was approximately 24.5 kDa as determined by SDS-PAGE.

Nucleic acid isolated from virus particles was infectious when inoculated on White Burley tobacco without any further treatment or when treated with DNase. Infectivity was, however, lost after treatment with RNase and S_1 nuclease, which showed ssRNA as the infectious entity. Electrophoresis of nucleic acid preparation revealed five major bands that moved along with two CMV strains isolated from *Physalis minima* L. and *Petunia* × *hybrida* Hort. Vilm.-Andr. (18). By analogy to other cucumoviruses, the fastest-moving band, which was near the bromophenol blue front, may be a satellite RNA.

In ODDT, crude antigen as well as purified virus preparation prepared from LPC were tested. Both preparations reacted strongly with antisera to CMV-L, CMV-C, CMV-T, and CMV-S. No reaction was observed with these antigens and antisera to either TAV-N or CAV (PVAS-24). In a separate experiment, virus isolate from chrysanthemum, two other strains of CMV, and a CAV isolate were tested for reaction with antisera to CAV (PVAS-24). In this test, CAV antisera did react with the CAV isolate but not with other CMV strains, including chrysanthemum isolate (Fig. 2). Chrysanthemum isolate, as well as three other CMV strains, did react with antisera to CMV-T, but under similar conditions CAV did not react with CMV-T antiserum (Fig. 3). Results of DAC-ELISA confirmed the findings of ODDT (Table 2). CMV-D antiserum, which was not included in ODDT, showed strong reaction with the chrysanthemum isolate.

DISCUSSION

Transmission of the virus isolate from chrysanthemums in a nonpersistent manner by aphids, the presence of infectious spherical particles of 29 nm in the purified preparation, the estimated molecular weight of viral protein subunits as 24.5 kDa by SDS-PAGE, loss of infectivity of nucleic acid by treatment with RNase but not with DNase, and separation of viral nucleic acid species as five major bands after electrophoresis in 1.2% agarose gel indicate the involvement of a satellite RNA containing cucumovirus with the diffused chlorosis symptoms in chrysanthemums.

In the Ouchterlony double-diffusion test, as in DAC-ELISA, the isolate reacted with antisera to CMV strains (CMV-L, CMV-C, CMV-T, CMV-S, and CMV-D [not tested in ODDT]) but not with antisera to CAV (PVAS) and TAV-N. The isolate thus appears to be a strain of CMV. No serological test with peanut stunt virus (another member of a cucumovirus group) was, however, conducted.

The isolate causes systemic mosaic on cucumber but does not induce enations on *N. tabacum* 'White Burley,' *N. tabacum* 'Samsun NN,' or on *N. rustica* L. like other CMV isolates. On the contrary, it induces systemic necrosis and death of the White Burley tobacco and local necrosis in some other tobacco species (Table 1). Incidentally, a CMV strain causing lethal necrosis in tobacco has not been recorded earlier. However, veinal necrosis in tobacco has been reported by a CMV strain infecting spinach (1). The strain has been identified as CMV-CI (C stands for chrysanthemum, and I for India). The necrosis in tobacco may be associated with the putative satellite RNA (CARNA 5) encapsidated in the virus particles. Currently, we are investigating the role of RNA in symptom production in tobacco.

Earlier records (9) show that chrysanthemum is not a host of CMV, and that it is occasionally used as a diagnostic host for differentiation between CMV and TAV. Existence of a CMV strain capable of infecting chrysanthemums is thus an important observation. Chrysanthemum, a popular ornamental plant propagated through suckers, might act as a reservoir of CMV-CI by providing inoculum to other economically important plants susceptible to CMV strains, especially those grown in kitchen gardens in India.

ACKNOWLEDGMENTS

We thank the following individuals for antisera: D. V. R. Reddy, ICRISAT, Hyderabad, India, for CMV-L; R. I. B. Francki, University of Adelaide, Australia, for CMV-T and TAV-N; A. A. Zaidi, CSIR Complex, Palanpur, India, for CMV-C, CMV-D, and CMV-S, and who in turn received it from H. A. Scott of the United States. Antiserum to CAV (PVAS-24) was purchased from the American Type Culture Collection.

Table 2. Serological relationship of the virus isolate from *Chrysanthemum* × *morifolium* with some known strains of cucumber mosaic virus (CMV) and tomato aspermy virus (TAV), as revealed by ELISA

	Optical density at 405 nm			
	Replicate			
Antisera[a]	I	II	III	Average
CAV	0.061	0.050	0.064	0.058 ± 0.007
TAV-N	0.002	0.002	0.002	0.002 ± 0.0
CMV-C	0.386	0.376	0.400	0.387 ± 0.012
CMV-D	0.426	0.418	0.430	0.425 ± 0.006
CMV-T	0.296	0.284	0.302	0.294 ± 0.009
CMV-L	0.308	0.302	0.326	0.312 ± 0.012
CMV-S	0.220	0.218	0.228	0.222 ± 0.005
BYMV	0.004	0.002	0.004	0.003 ± 0.001

[a]CAV = chrysanthemum aspermy virus (PVAS-24, ATCC), TAV-N = tomato aspermy virus, CMV = cucumber mosaic virus, BYMV = bean yellow mosaic virus.

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