CHARACTERIZATION OF AN INDIAN ISOLATE OF CUCUMBER MOSAIC VIRUS INFECTING EGYPTIAN HENBANE (*HYOSCYAMUS MUTICUS* L.)

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Summary. – A cucumber mosaic virus isolate was found to be associated with mottle crinkle and severe mosaic disease of Egyptian henbane (*Hyoscyamus muticus* L.). The virus has been characterized as an Indian isolate of cucumber mosaic virus (CMV) based on non-persistent transmission by aphid, presence of 28-nm isometric particles, capsid protein of 26 K and single-stranded tripartite RNA genome with a subgenomic RNA (RNA 4). There was no evidence of satellite RNA genome. The isolate showed a strong serological relationship with S and A strains of CMV (CMV-S and CMV-A) in double diffusion test. A band of the 26 K capsid protein was also detected by Western blot analysis using antibodies specific to CMV-S.

Key words: cucumber mosaic virus; Hyoscyamus muticus; mottle crinkle; severe mosaic; Western blot analysis

Introduction

Egyptian henbane (*Hyoscyamus muticus* L.), a member of *Solanaceae* family and commonly known as Egyptian henbane, is cultivated in different parts of the world as an ideal raw material for hyoscyamine and hyoscine alkaloids. The leaves and flowers contain 1% of alkaloids of the tropane group, mainly hyoscyamine, hyoscine and atropine (Hocking, 1955). These alkaloids are used in various modern drugs, which are in great demand in Europe and northern Asia over a long period of time. Hyoscyamine is used as a sedative and antispasmodic and in the control of gripping pain in intestinal disorders. Hyoscine is also used in paralysis agitants, cough, asthma and also in the control of excitement in cases of nervous disorders (Chopra *et al.*, 1958).

A mottle crinkle and severe mosaic disease of *H. muticus* was noticed in the experimental fields of CIMAP, Lucknow, during the months of April and May in 1996–1998. The disease was severe and fairly wide spreading. A literature survey on the viral diseases affecting *H. muticus* revealed that the single, double and triple infections with potato virus X, cucumber mosaic virus and tobacco rattle virus reduced fresh weight, length of infected plants and their content of total alkaloids (El-Hammady *et al.*, 1982). Subba Rao and Akhtar Husain (1984) reported a green mosaic disease of *H. muticus* and suspected it to be caused by infection with cucumber mosaic virus but the real causal agent was not identified. Affiliation of tomato spotted wilt virus with *H. muticus* was also recorded (Zaim, 1989).

Association of a potyvirus and crystalline inclusion bodies with a mosaic disease of another species of henbane (*H. niger* L.) have been reported from time to time and worked out by several authors (Hamilton, 1932; Chenulu *et al.*, 1968; Govier and Plumb, 1972; Stefanac *et al.*, 1993). However, detailed studies characterizing the virus causing

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Abbreviations: CMV = cucumber mosaic virus; DEPC = diethyl pyrocarbonate; dsRNA = double-stranded RNA; p.i. = post inoculation; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TAV = tomato aspermy virus

mottle crinkle and severe mosaic disease of *H. muticus* have not been done so far in India.

Thus, we attempted here to characterize the virus isolate by electron microscopy, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) of coat protein subunits, nucleic acid analysis, double diffusion test and Western blot analysis.

Materials and Methods

Mechanical transmission and host range experiments. Leaf sap from naturally infected *H. muticus* leaf tissues prepared in 0.1 mol/l phosphate buffer pH 7.0 was used for mechanical inoculation. The seedlings of various plant species belonging to different families (Table 1) were inoculated. The inoculated plants were maintained and observed for one month for the appearance of symptoms. Back inoculation on *Chenopodium amaranticolor* was done to determine the presence or absence of the virus in the plants, which remained symptomless after one month post inoculation (p.i.).

Aphid transmission. Aphids *Myzus persicae* (Sulzer) and *Aphis gossypii* (Glover) were used for aphid transmission of the virus as described earlier (Raj *et al.*, 1995). After a starvation period (1–2 hrs) the aphids were allowed to probe on infected *H. muticus* plants (1–2 mins) and were then placed on healthy *H. muticus* and *Nicotiana rustica* plants for 24 hrs. Aphids were sacrificed by spraying with 0.02% Rogor. The plants were observed for one month to observe virus symptoms and tested by back inoculations.

Table 1. Symptoms appearing on various species after virus inoculation

Plant species	Local symptoms (3–6 days p.i.)	Systemic symptoms (15–20 days p.i.)
Amaranthus hypochondriachus	CLL	SM
Capsicum annuum	–	–
Chenopodium album	NLL	–
C. amaranticolor	NLL	–
C. murale	NLL	–
Cucumis sativus	–	SM
Cucurbita pepo	–	SM
Datura stramonium	CLL, LS	SM, LD
Hyoscyamus muticus	–	SM, LD
Lycopersicon esculentum	–	SM, LD
Nicotiana benthamiana	–	SM, ST, LD
N. glutinosa	NLL	SM
N. rustica	NLL	SM
N. tabacum cv. DR1	NLL	SM
N. tabacum cv.Harrison Special	NLL, NR	MM
N. tabacum cv. Samsun NN	CLL	SM
N. tabacum cv. White Burley	CLL	SM, LC
N. tabacum cv. Xanthi	CLL	SM

CLL = chlorotic local lesions, LD = leaf deformations, LS = leaf shedding, MM = mild mosaic, NLL = necrotic local lesions, NR = necrotic rings, SM = severe mosaic, ST = stunting of growth, (–) = no symptoms.

Virus purification, UV spectrum and electron microscopy. The virus isolate was purified from 100 g of inoculated leaves of *N. tabacum* cv. Xanthi according to the procedure of Lot *et al.* (1972). Briefly, infected leaf tissues harvested 7–10 days p.i. were ground in 300 ml of the extraction buffer A (500 mmol/l sodium citrate buffer pH 6.5, 5 mmol/l EDTA, and 5 g/l thioglycolic acid), squeezed through two layers of muslin cloth, and the crude extract was mixed with chilled chloroform, stirred for 30 mins and centrifuged for 10 mins at 10,000 rpm at 4°C. The aqueous supernatant was loaded on 150 g/l sucrose cushion and centrifuged for 2 hrs in at 35,000 rpm a Beckman Ti 45 rotor at 4°C. The obtained pellet was suspended in 10 ml of the B buffer (5 mmol/l sodium borate pH 9.0, 0.5 mmol/l EDTA, and 10 ml/l Triton-X 100), stirred overnight at 4°C, clarified by low speed centrifugation and pelleted by high speed centrifugation in a Beckman Ti 50 rotor at 40,000 rpm for 2 hrs. The final pellet was resuspended in 1 ml of the B buffer without Triton-X 100 and subjected to UV spectrum analysis, electron microscopy and inoculations on *C. amaranticolor* to assess the presence of virus particles examined the purified virus preparations. The yield of virus was calculated by assuming the extinction coefficient 5 at 260 nm (Francki *et al.*, 1979). For electron microscopy, virus preparations were stained with uranyl acetate (20 g/l pH 4.2) and examined under a Philips EM 420 transmission electron microscope.

Serological studies. To establish serological relationship between viruses or virus isolates, a double diffusion test was carried out. Crude antigens for the double diffusion test were prepared by grinding infected leaf tissue in 10 mmol/l phosphate buffer pH 7.0 in the ratio of 1:2 (w/w). The central well was charged with crude antigen and peripheral wells with antisera to various viruses or virus isolates (CMV-S, CMV-T, CMV-CD, CMV-P, CMV-A and tomato aspermy virus (TAV)) diluted 1:10 and incubated overnight at room temperature (28–30°C) in a humid chamber. Original antisera to various CMV strains (CMV-S (PVAS 242a) and CMV-CD (PVAS-30), and TAV (PVAS-24) were purchased from American Type Culture Collection (ATCC), USA, and CMV-T was a gift from Dr. R.I.B. Francki, Waite Agricultural Research Institute, Adelaide, Australia. The antisera to CMV-A (Raj *et al*, 1996) and CMV-P (Haq *et al*, 1996) were raised in our laboratory.

SDS-PAGE of viral protein subunits and Western blot analysis. To determine M_r of coat protein subunits a 15 µl sample of purified virus preparation was boiled in a dissociation buffer for 3 mins, then loaded on 120 g/l acrylamide gel and electrophoresed as described earlier (Maizel, 1971). M_r of viral coat protein subunits was estimated by comparing their relative mobilities to those of the standards (Sigma). After SDS-PAGE, protein bands were electroblotted on a nitrocellulose membrane and Western blot analysis was performed as described by Renart and Sandoval (1984). Antibodies to CMV-S (PVAS 242a, 1:500) and antirabbit IgG alkaline phosphatase conjugate (Sigma) were used at 1:1000 dilutions.

Nucleic acid extraction and analysis. Viral nucleic acid was extracted by disrupting the purified particles of CMV with equal volumes of 10 g/l SDS and phenol/chloroform and followed by ethanol precipitation (Srivastava *et al.*, 1992). Nucleic acid preparations in DEPC-treated water were treated with DNase and

RNase separately at 37°C for 30 mins and their infectivity was checked by inoculating them on *C. amaranticolor*. For separation of RNA species, electrophoresis was carried out in 12 g/l agarose gel under non-denaturing conditions as described earlier (Sambrook *et al.*, 1989). The gel was stained with 0.5 mg/ml ethidium bromide and visualized under UV light. A double-stranded RNA (dsRNA) was isolated from experimentally inoculated and healthy *N. tabacum* cv. Xanthi leaves according to the protocol of Diaz-Ruiz and Kaper (1978) and analyzed in 20 g/l agarose gel electrophoresis. Lambda DNA double digested with *Eco*RI and *Hind*III from Bangalore Genei was used as molecular size marker.

Results

Mechanical transmission and host range

Naturally infected Egyptian henbane (*H. muticus* L.) plants exhibited severe mosaic, leaf deformations, puckering of leaves and stunting symptoms (Fig. 1). Infected plants also produced smaller leaves, reduced number of flowers, and poor seed setting (as compared to healthy). A premature death of infected plants due to drying of whole plant was noticed during 1996–1998.

Experimental sap inoculations on various plants revealed that virus was easily transmitted from *H. muticus* to *H. muticus* and a number of host species (Table 1). *H. muticus* plants produced systemic symptoms very similar to the natural ones within 15–20 days p.i. The inoculated *Amaranthus hypochondriachus, C. album, C. amaranticolor C. murale, Cucumis sativus, Cucurbita pepo, Datura stramonium, Lycopersicon esculentum, N. benthamiana, N. glutinosa, N. rustica,* and *N. tabacum* cv. DR-1, Harrison Special, Samsun NN, White Burley, and Xanthi showed various kinds of local and systemic symptoms. However, neither local and/or systemic symptoms appeared on *Capsicum annuum, Cucumis melo, Dolichos lablab, Laginaria vulgaris, Luffa cylindrica, Ocimum basilicum, Physalis minima, Solanum melongena, Vicia faba* and *Vigna radiata* nor virus could be detected in them by back inoculation on *C. amaranticolor* indicator host.

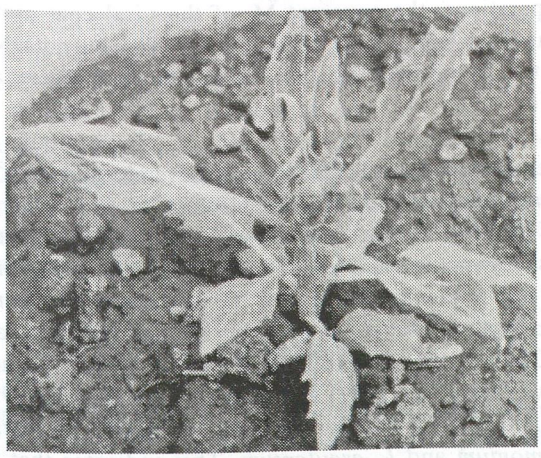

Fig. 1
Naturally infected Egyptian henbane (*H. muticus* L.) exhibiting severe mosaic, leaf deformations and stunting symptoms

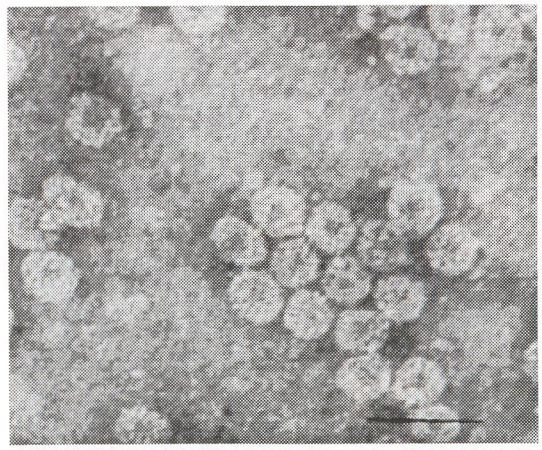

Fig. 2
Electron micrograph of purified virus preparation showing isometric particles of 28 nm with a central core
Magnification 228,000x. Bar = 56 nm.

Aphid transmission

Aphid transmission tests revealed that *M. persicae* and *Aphis gossypii* successfully transmitted the virus in a non-persistent manner from *H. muticus* to *H. muticus* (3/5) and *N. rustica* (4/5) plants. The symptoms were identical to those obtained after sap inoculation.

Virus purification, UV absorption spectrum and electron microscopy

Purification of virus preparation from systemically infected *N. tabacum* c.v. Xanthi leaves gave a virus yield of 150 mg/kg of fresh tissue and a typical UV absorption spectrum of nucleoprotein with an absorbance of $A_{260/280}$ ratio of 1.7. Electron microscopy of purified virus preparations revealed polyhedral virus particles of 28 nm in diameter with a clear central core (Fig. 2). The shape and size of the virus particles were identical to those of CMV particle (Francki *et al.*, 1979).

Serological studies

The double diffusion test showed a strong, moderate and weak reaction of the virus isolate with the antisera to CMV-S

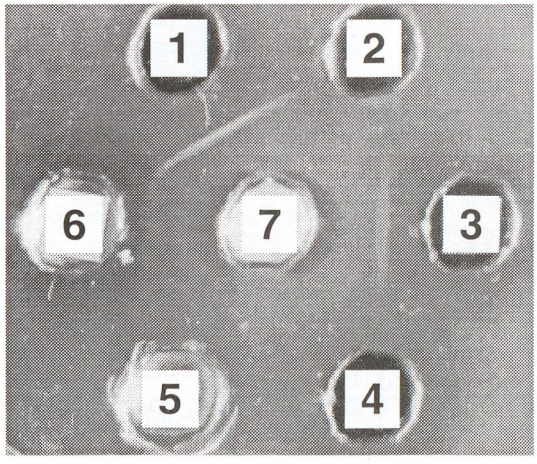

Fig. 3
Homologous gel diffusion test
Central well (7): crude sap from naturally infected *H. muticus* leaf. Peripheral wells: antisera to CMV-S (PVAS 242a) (1), CMV-CD (2), CMV-A (3), TAV (4), CMV-P (5), and CMV-T (6).

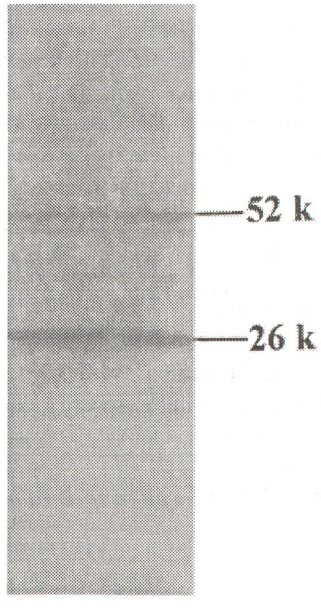

Fig. 4
Western blot analysis using antiserum to CMV-S (PVAS 242a)
Hybridization as 2 bands of 26 K and 52 K (dimer) capsid protein of CMV isolate from *H. muticus*.

(PVAS 242a), CMV-A and CMV-T (Francki) strains, respectively. The virus isolate showed a close relationship to CMV-S and CMV-A (Raj *et al.*, 1996) and a distant relationship to CMV-T but not to CMV-CD, CMV-P and TAV (Fig. 3).

SDS-PAGE of viral protein subunits and Western blot analysis

SDS-PAGE and Western blot analysis revealed the presence of one major band of about 26 K identical to the CMV coat protein and a minor band of about 54 K, which seems to be a dimer of the 26 K protein. In Western blot analysis using antibodies to CMV-S (PVAS 242a) the both bands of the same size were observed as in the case of SDS-PAGE (Fig. 4).

Nucleic acid extraction and analysis

Nucleic acid preparations obtained from purified virus particles remained infectious on *C. amaranticolor* even when treated with Dnase, but the infectivity was completely lost by treatment with RNase and S1 nuclease indicating the presence of single-stranded RNA (ssRNA) in the virions. In agarose gel electrophoresis of RNA preparations, 3 distinct bands were observed corresponding to RNA 1 plus RNA 2, RNA 3, and RNA 4. There was no evidence of satellite RNA (Fig. 5).

The dsRNA extracted from infected *N. tabacum* was separated into 3 distinct bands by agarose gel electrophoresis, corresponding to 3 different genomic ssRNAs:RNA 1 (M_r of 2.56×10^6), RNA 2 (2.24×10^6) and RNA 3 (1.66×10^6). No such bands were observed in a similarly extracted dsRNA from healthy plants (Fig. 6). The pattern of dsRNA separation resembles that found earlier in the case of bananas infected with CMV (Kiranmai *et al.*, 1996).

Discussion

Virus transmission in non-persistent manner by aphids, presence of isometric particles of 28–29 nm in the purified preparations, the apparent M_r of the viral coat protein subunits of 26 K, loss of infectivity by RNase but not DNase treatment, and separation of viral nucleic acid species as 3 major bands after electrophoresis in 1.2% agarose gel indicate the involvement of CMV in crinkle mottle and severe mosaic disease of *H. muticus*. Several reports confirm these findings (Palukaitis *et al.*, 1992).

The CMV isolate from *H. muticus* caused systemic mosaic symptoms and leaf deformations on *D. stramonium and L. esculentum* but not on *C. annuum* and *Solanum melongena*. However, in our earlier studies, Indian isolates of CMV from amaranth (Raj *et al.*, 1996), petunia (Srivastava *et al.*, 1991) and carnations (Raj *et al.*, 1993) did not produce local or systemic symptoms on *Datura stramonium* and *L. esculentum*. This indicates that the present isolate is biologically different from the reported Indian isolates.

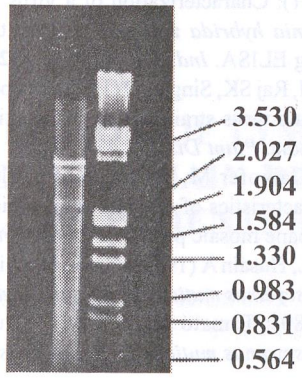

Fig. 5
Agarose gel electrophoresis of dsRNA isolated from *H. muticus* leaves
Healthy (lane A), infected (lane B) and Lambda DNA double digest with *Eco*RI/*Hind*III (lane C) as DNA marker from Bangalore Genei.

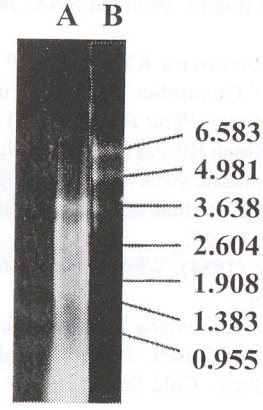

Fig. 6
Agarose gel electrophoresis of RNA isolated from purified virus isolate
Purified virus isolate (lane A), RNA size marker (lane B).

In the double diffusion test the virus isolate reacted with the antisera to CMV-S, CMV-A and CMV-T but not to TAV. On the basis of positive reaction in the double diffusion test and the presence of 26 K protein band in Western blot analysis using antibodies to CMV, the virus isolate appears to be CMV.

Since dsRNA analysis is one of the diagnostic characteristics for identification of ssRNA viruses (Dodds, 1993), several isolates of CMV have been distinguished on the basis of dsRNA separation in agarose gels by electrophoresis (Pares *et al.*, 1992). Our observations revealed the separation of dsRNA as three bands corresponding to the size of three genomic species of CMV ssRNA which indicates that the virus isolate is CMV.

A green mosaic disease of *H. muticus* was studied by Subba Rao and Husain (1984) and based on preliminary observations such as host range and biophysical properties, a CMV infection was suspected. However, our findings confirm the association of CMV with mottle crinkle and severe mosaic disease of *H. muticus*.

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