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Biological and N–Terminal Serological Properties of a Strain of Henbane Mosaic Virus Causing Mosaic Disease of *Datura metel* Linn.

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ABSTRACT. *A mechanically transmissible flexuous virus causing severe mosaic with leaf blisters and filiformy of Datura metel was identified as a strain of henbane mosaic potyvirus (HMV–Da) on the basis of its host range, vector transmissibility, electron–microscopy and N–terminal serology. The virus was restricted to species of Solanaceae and induced characteristic systemic symptoms in Hyoscyamus niger and Nicotiana spp. but it was apathogenic to Capsicum annum, Lycopersicon esculentum, Solanum spp.. The virus was vectored by aphids, Aphis gossypii Glover and Myzus persicae Sulzer in a non persistent manner. Virus particles measuring 795 × 12 nm and cytoplasmic cylindrical inclusions of pinwheels, scrolls and curved laminated aggregates with elongated mitochondria, characteristics of HMV, were observed in infected tissues. The virus coat protein (~ 30 KD) strongly reacted with polyclonal antiserum to HMV directed towards intact coat protein in DAC–ELISA. The identity of the virus was further confirmed as a strain of HMV by using polyclonal antiserum directed towards the N–terminal region.*

INTRODUCTION

Datura metel is an important medicinal plant with antiviral property (Verma *et al.*, 1995). The alkaloids hyoscine, hyoscyamine and daturin extracted from its plant parts are known to have narcotic and antispasmodic properties. Its use as an oral contraceptive by the Saora tribals of Orissa has also been shown (Prem Kishore *et al.*, 1982). It serves as a reservoir of a large number of viruses (Horvath *et al.*, 1988). In India, Datura has been found commonly infected with a virus disease characterized by severe systemic mosaic with leaf blisters and filiformy. Association of seven different viruses, such as datura virus 3 (Capoor and Verma, 1948, 1951), datura virus 3A (Garga, 1958), datura virus 3B (Yaraguntaiah and Govindu, 1972), datura enation mosaic virus (Verma and Verma, 1963), potato virus Y (Rao and

Yaraguntaiah, 1976), datura mosaic virus (Qureshi and Mahmood, 1978) and datura distortion mosaic virus (Mali *et al.*, 1985; Gahukar and Patil, 1991; Prasanna *et al.*, 1996) from different localities has been recorded. Despite inadequate characterization, these viruses have been named differently by various workers. We have studied a potyvirus associated with a mosaic disease of Datura from Delhi and Aligarh and have established its relationship to henbane mosaic virus (HMV) strain by biological and N–terminal serological studies.

MATERIALS AND METHODS

Virus sources and maintenance

The datura plants showing yellowish green, blistered and malformed foliage were observed along road side in Delhi. The virus infected plants was extracted in 0.05 M borate buffer and sap inoculated on to 10 day old seedlings of *Nicotiana glutinosa* Linn. and *N. tabacum* var White Burley. Symptoms constituting of severe, mosaic, with blistered, malformed leaves were observed in both the hosts. The virus, was hence maintained in *N. glutinosa* and *N. tabacum* and was designated DaMV–DL. Another isolate designated as DaMV–AL producing similar symptoms was obtained from Aligarh Muslim University and was also investigated.

Host range and symptomatology

Ten healthy seedlings each of *Arachis hypogaea* L., *Capsicum annum* L., *Chenopodium amaranticolor* L., *Cucumis sativus* L., *Cucurbita moschata* (Duch) Poir, *Glycine max* Merr., *Hyoscyamus niger* L., *Luffa acutangula* Roxb., *Lycopersicon esculentum* Mill., *Nicotinana glutinosa* Linn., *N. tabacum* Linn. *cv.* Samsun, *N. tabacum* Linn. *cv.* Xanthi, *Petunia hybrida* L., *Solanum melongena*, L., *S. tuberosum* L., *Vigna radiata* Roxb., *V. mungo* Hepper and *V. unguiculata* were mechanically inoculated with standard inoculum of both the isolates. The test plants were placed in an insect proof glass house, observed for symptoms for 3–4 weeks and tested for virus infection by leaf–dip electron microscopy and DAC–ELISA.

Insect transmission

Two different aphid species *viz. Aphis gossypii* Glover and *Myzus persicae* Sulzer were evaluated for their efficiency as vectors. Aviruliferous aphids were given a starvation period of 2 h followed by an acquisition – and inoculation access period of 10 min and 24 h respectively. Ten plants of *N. glutinosa*/*N. tabacum* cultivar Xanthi were used for tests with each aphid species (10 viruliferous aphids/plant).

Cytopathology

Infected samples were subjected to double fixation by glutaraldehyde [2.5% (v/v) in 0.1 M phosphate buffer, pH 7.0] followed by osmium tetraoxide [0.2% (v/v) in 0.01 M phosphate buffer, pH 7.0]. After dehydration in graded dilution of acetone, they were embedded in a mixture of propylene oxide and resin and finally incubated overnight at 60°C in an oven. The ultra–thin sections were stained with uranyl acetate and lead citrate and were finally examined under JEOL 100 CX II electron microscope.

Virus purification and polyclonal antiserum production

Virus was purified using Moghal and Francki's (1976) procedure with certain modifications. The infected leaf tissues were extracted in 0.05 M borate buffer and the expressate emulsified with equal volume of carbon tetrachloride and chloroform (1:1 v/v). The mixture was then spun at 6,600 g for 10 min in a Sorvall RC–5C SS–34 rotor. After clarifying the supernatant through Whatman No. 1 filter paper, polyethylene glycol (PEG 6000 4%, v/v) and sodium chloride (1.75%, v/v) were added. After incubating the mixture for 1 h at 4°C, virus particles were sedimented by centrifugation at 6,600 g for 10 min. Virus pelletization was done at 100850 g through a 10 ml sucrose pad (30% w/v) in a Beckman 55.2 Ti rotor for 90 min. Gradient centrifugation was carried out on a linear gradient of 20–50% (w/v) sucrose, at 151200 g in Beckman SW 41 Ti rotor for 90 min. Final pelletizing of the virus particles was done at 100850 g in Beckman 552 Ti rotor for 90 min.

The purified virus was scanned in CIBA Corning, 2800 spectrascan spectrophotometer from 200–300 nm. Absorbance ratio 260:280 and virus yields were estimated by assuming extinction coefficient (EC) of a potyvirus as 2.5.

For preparing the antiserum, rabbits were immunized with freshly purified virus preparation (500 μg per injection) emulsified with Freundt's incomplete adjuvant (1:1 v/v) intramuscularly at weekly intervals. After four such injections, the rabbit was bled two weeks after the last injection and thereafter at weekly intervals. About 15 ml serum was collected each time, allowed to coagulate at room temperature and kept at 4°C overnight. The clear antiserum obtained after a low speed centrifugation at 6,600 g for 5 minutes was mixed with an equal volume of glycerol and stored at –20°C. The antisera titre was determined by DAC–ELISA test.

Polyacrylamide gel electrophoresis

Molecular weight (Mw) of coat protein was determined by SDS–PAGE according to the method of Laemmli (1970). Samples were mixed with an equal volume of sample buffer, heated for 3 min in boiling water and electrophoresed in 5% stacking gel (1.2 cm) and 12% resolving gel (10 cm). After electrophoresis, the gels were stained with Coommassie Brilliant Blue R–250 (Sigma, USA). Low range protein molecular weight markers used were Carboni anhydrase (Mw 31K), Soybean trypsin inhibitor doublet (Mw 20, 4K) Horse heart myoglobin (Mw 16.9K), lysozyme (Mw 14.4K), myoglobin (F_1) (Mw 8.1K), myoglobin (F_2) (Mw 6.2K) and myoglobin (F_3) (Mw 2.5 K).

Serology

Serological studies were performed using direct antigen coated–enzyme linked immunosorbent assay (DAC–ELISA), Western blotting (WB) and immunosorbent electron microscopy (ISEM) tests. Polyclonal antisera directed towards intact coat protein of cowpea–aphid borne mosaic (CABMV), egg plant mottle (EMoV), henbane mosaic (HMV), potato virus Y (PVY) and watermelon mosaic virus–1 (WMV–1) and N–terminal region of HMV coat protein obtained from Advanced Centre for Plant Virology collection, IARI, New Delhi were used after cross absorption.

DAC–ELISA was done in polystyrene plates using the protocol described by Clark and Bar–Joseph (1984). Plant antigens were prepared by extracting fresh leaf tissues in carbonate buffer (1:10 w/v) containing PVP (2%, w/v, Mw 40,000). The clear supernatant was used as viral antigens. The plates were first coated with antigens for 1 h at 37°C. After washing in PBS–T and blocking in 5% (w/v) milk powder solution, antiviral antibody in PBS–T was

added in 1:500 dilution and incubated for 2 h at 37°C. The plates were then washed with PBS–T (2–3 min each) and incubated in antirabbit immunoglobulin–alkaline phosphatase conjugate (1:6000). The reaction of ELISA were read at 405 nm after adding the substrate (0.5 mg/ml paranitrophenyl phosphate).

WB was performed after SDS–PAGE as described by O'Donnell *et al.* (1982). The protein bands were transferred to nitrocellulose membrane and reacted with anitbody followed by alkaline phosphatase conjugated secondary anitbodies for 1 h each. A mixture of nitro blue tetrazolium (NBT) and bromo–chloroindolylphosphate (BCIP) was used as substrate.

Immunosorbent electron microscopy (ISEM) decoration test was conducted as described by Milne and Lesemann (1984). The antiserum at dilution of 1:70 was used for decoration of virus particles. The incubation period was 20–25 min.

RESULTS

Insect transmission

A. gosspyii transmitted both DaMV–DL and DaMV–AL isolates to 6 of 10 (60%) and 4 of 10 (40%) *N. tabacum cv.* Xanthi plants, respectively. However, *M. persicae* transmitted only one of the isolates *i.e.* DaMV–DL to 4 of 10 (40%) *N. tabacum cv.* Xanthi and *N. glutinosa* plants. All symptomatic plants were virus infected as verified by DAC–ELISA.

Host range and symptomatology

N. glutinosa developed systemic symptoms consisting of vein clearing, chlorotic spots, reduction of lamina, yellowing and stunting. On *N. tabacum cvs.* Samsun and Xanthi, the reactions were vein clearing, mosaic, puckering and blistering. *H. niger* that was systemically infected exhibited vein clearing, mosaic and blistering. All symptomatic plants were virus infected as verified by DAC–ELISA. The plant species which were not infected and from which no virus was recovered were : *Arachis hypogaea* L., *Capsicum annum* L., *Chenopodium amaranticolor* L., *Cucumis sativus* L., *Cucurbita moschata*

(Duch.) Poir, *Glycine max* Merr., *Luffa acutangula* Roxb., *Lycopersicon esculentum* Mill., *Petunia hybrida* L., *Solanum melongena* L., *S. tuberosum*, *Vigna radiata* Roxb., *V. mungo* Hepper and *V. unguiculata.*

Table 1. Species of Solanaceae susceptible to DaMV–AL and DaMV–DL isolates.

Species[1]	Reactions[2]		Symptoms[3,4]	
	DaMV–AL	DaMV–DL	DaMV–AL	DaMV–DL
Hyoscyamus niger L.	S	S	B,M,VC	B,M,VC
N. glutinosa	S	S	CS,LR,S,VC,Y	CS,LR,S,VC,Y
N. tabacum L.				
cv. Samsun	S	S	B,M,P,VC	B,M,P,VC
cv. White Burley	S	S	B,M,P,VC	B,M,P,VC
cv. Xanthi	S	S	B,M,P,VC	B,M,P,VC

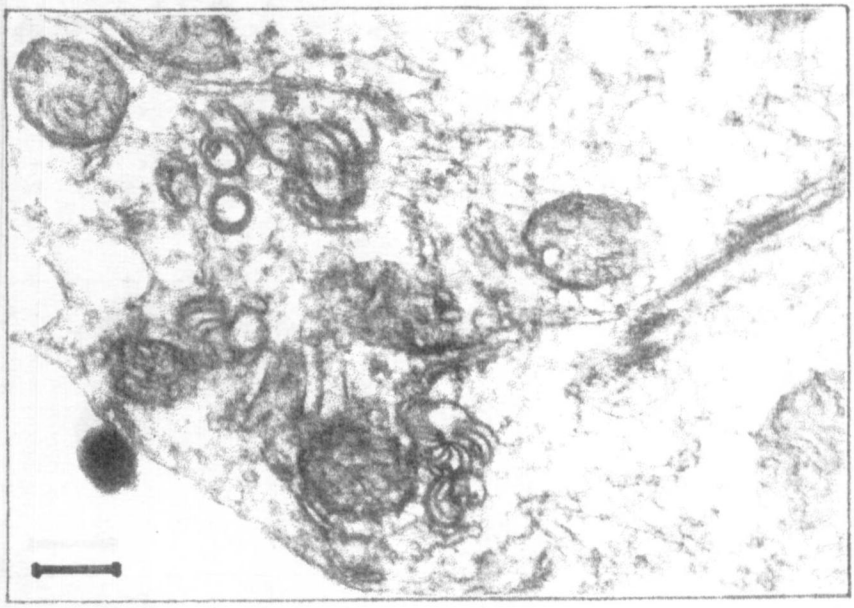
1. At least, 5 plants of each species were inoculated mechanically
2. S = Susceptible
3. B = Blistering; CS = chlorotic spots; M = Mosaic; LR = Lamina reduction; P = puckering; S = Stunting; VC = Vein clearing; Y = Yellowing
4. Symptomatic plants were checked for the virus by DAC–ELISA.

Cytopathology

Inclusions with varying configurations such as pinwheels, scrolls and laminated aggregates with elongated mitochondria were observed in infected tissues (Figure 1).

Virus purification

The absorption spectrum of purified DaMV–DL and DaMV–AL isolates were typical of nucleoprotein, with maxima around 260 nm and minima around 245 nm. The ratio of absorbance (260:280) was 1.08. Based on



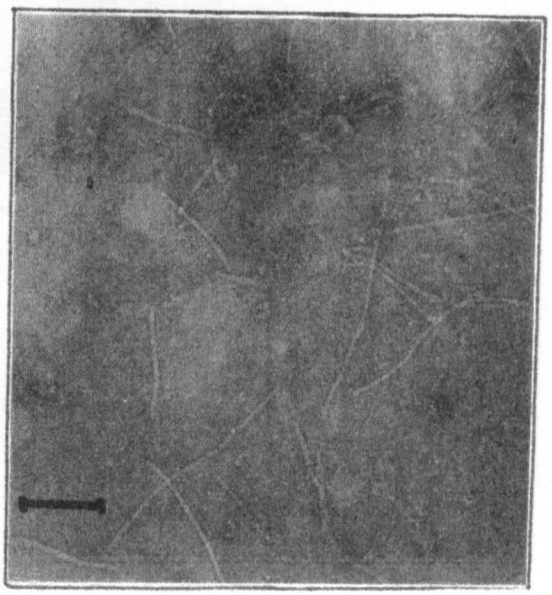
(Bar represents 350 nm)

Figure 1. Infected leaf cells of Datura showing pinwheels, scrolls, and elongated mitochondria.

extinction coefficient value given for the potyvirus, the yield of purified virus (Figure 2) was calculated to be 3.896×10^2 ng/100 g of leaf material. The mean and modal length of DaMV–DL was found to be 776 and 795, respectively (Figure 3). There was a close parity in the observation of DaMV–AL regarding the mean and model length. In purified preparation, no end to end aggregation of the particles was observed.

Virus coat protein

The coat of DaMV–DL and DaMV–AL had one major band that ran along with the purified coat protein of HMV and had an apparent molecular weight of 30,000 Daltons.



(Bar represents 125 nm)

Figure 2. **Purified virus particles of DaMV–DL.**

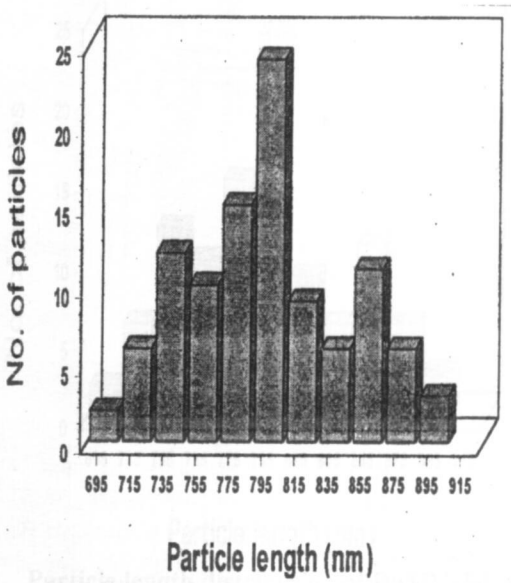


Figure 3. **Particle length distribution of DaMV–DL in leaf–dip preparation.**

Serology

Of the five polyclonal antisera directed against intact coat protein tested in DAC–ELISA, DaMV–DL and DaMV–AL isolates strongly reacted with HMV antiserum showing A_{405} value of 0.5 to 0.7 besides their homologous antiserum. The DaMV–DL and DaMV–AL reacted only moderately as revealed by A_{405} value of 0.3 to 0.5 with polyclonal antiserum to PVY. However, it reacted very weakly with polyclonal antisera to CABMV, EMoV and WMV–1 (A_{405} value ranging from 0.1 to 0.3). The virus isolates also strongly reacted with polyclonal HMV antiserum directed towards the N–terminal region of the coat protein in DAC–ELISA. Positive cross reaction of DaMV–DL and DaMV–AL isolates with intact coat protein and N–terminal HMV antiserum was also observed in WB and ISEM tests (Figure 4).

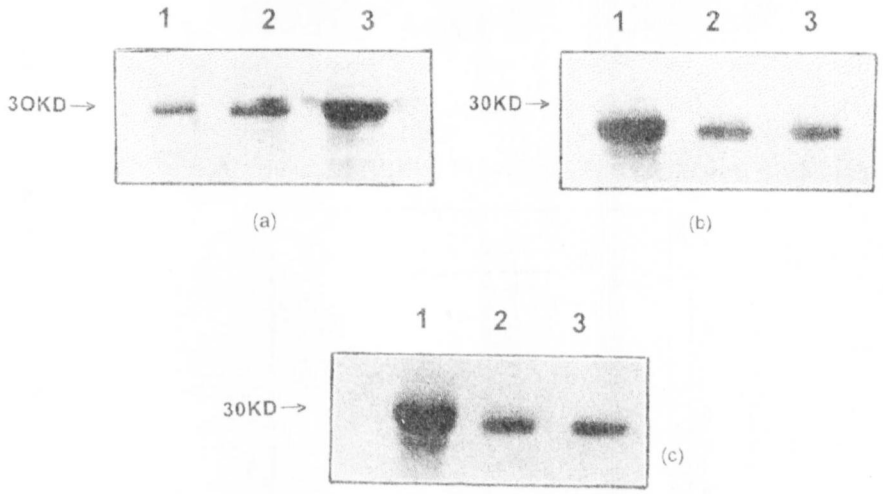


Figure 4. **SDS–PAGE (a) and Western blot of coat proteins of potyviruses (b,c). Lane 1–3 : DaMV–DL, DaMV–AL and HMV, respectively. a : stained with Coomassie Brilliant Blue R–250. b : probed with an antiserum to N–terminal region of HMV. c: probed with DaMV–DL antiserum.**

DISCUSSION

Several viruses have been reported to cause infection in Datura, resulting in severe symptoms like mosaic, blistering and malformation of leaf lamina leading to filiformy. Lack of adequate characterization caused confusion in terminology, as discussed earlier. In the present paper we attempt to identify a virus causing mosaic like symptoms in Datura at New Delhi and Aligarh. The viruses have been fully characterized, and their serological relationship with other members of potato virus Y have been investigated.

The virus isolates DaMV–DL and DaMV–AL are definitive members of "Potyviridae" as revealed by the flexuous filamentous particles measuring 795 × 12 nm with A (260:280) ratio of 1.08, which induced cytoplasmic inclusions of pinwheels, scrolls and laminated aggregates types, strongly reacted with antiserum of HMV and were vectored by aphids in a non–persistent fashion. The types strain of HMV has particle size of 800–900 nm (Govier and Plumb, 1972) and A (260:280) ratio of 1.1. Our isolates have the same range of particle size and A (260:280) ratio. They differ from potyviruses reported to naturally infect *Datura spp.* in India. For example, they differ from datura mosaic virus (Qureshi and Mahmood, 1978) in being aphid transmissible and not infecting *Petunia hybrida.* They are also different from PVY (Rao and Yaraguntaiah, 1976) and datura distortion mosaic virus (Mali *et al.*, 1985; Gahukar and Patil, 1991; Prasanna *et al.*, 1996) as they did not infect *Capsicum annum*, *Chenopodium amaranticolor*, *Petunia hybrida* and *Solanum tuberosum.*

The vector *Aphis gossypii* transmitted both isolates efficiently. However, the isolate from Aligarh was not transmitted by *M. persicae.* Host range determined by sap and vector transmission was similar to that of HMV (Govier and Plumb, 1972). The virus is identified as HMV as it strongly reacted with ploYclonal HMV antiserum directed towards intact coat protein in DAC–ELISA, Western blotting and ISEM tests. The identity of virus was further confirmed by using polyclonal HMV antiserum directed towards N–terminal region, as N–terminal is virus specific and viruses reacting with antibodies to N–terminal are strains of one virus (Shukla and Ward, 1989).

Based on host range studies, insect transmission, Cytopathology and serology, it was deduced that both DaMV–DL and DaMV–AL are identical to HMV and could thus be designated at HMV–Da. Natural infection of HMV on Datura (Lovisolo and Bartels, 1970) further supports our contention. However, in order to unequivocally identify DaMV–DL and DaMV–AL as strains of

HMV, coat proteins and 3' non–translated regions sequences need to be compared.

CONCLUSIONS

A virus causing mosaic disease of Datura was isolated from two isolates, DaMV–DL and DaMV–AL and identified as a member belonging to potato virus Y group. Based on particle morphology, host range and N–terminal serology the virus was identified as a distinct strain of HMV.

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