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Association of Poty and Carlaviruses with Mosaic Disease of *Hippeastrum stylossum*

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ABSTRACT. *A mosaic disease encountered on Hippeastrum stylossum was sap transmissible to Chenopodium amaranticolor and Hyoscyamus niger by producing local lesions and to Gomphrena globosa systemically. Aphis gossypii Glover., Brevicoryne brassicae L., Lipaphis erysimi Kalt. and Myzus persicae Sulz. failed to transmit the disease. Filamentous virus particles observed under an electron microscope were distributed in a range of 450–950 nm with two maxima, one at 600–650 nm and other at 750–800 nm. The purified virus preparation showed maximum absorbance at 260 nm and had coat protein of 27 and 33 kDa. Polyclonal antiserum was produced in rabbit which had a titre of 1:512 in tube precipitin test and 1:1024 in DAC–ELISA and ISEM tests. The purified virus preparation reacted strongly to antisera of carnation latent (CLV), shallot latent (SLV) carlaviruses and onion yellow dwarf (OYDV), potato Y (PVY) potyviruses in ELISA, ISEM and EBIA tests. These serodiagnostic methods not only detected the virus(es) but also showed the association of four viruses with hippeastrum mosaic disease which are hitherto unrecorded on this host.*

INTRODUCTION

The *Hippeastrum spp.*, commonly known as 'Dutch lily', belongs to the family *Amaryllidaceae* and occupies an important place in cutflower markets due to its large sized flowers of fascinating colours and good keeping quality. Natural occurrence of seven viruses on *Hippeastrum spp.* has been reported from different countries which cause moderate to severe losses to this ornamental crop. They are; tomato spotted wilt virus (TSWV; Smith, 1935), cucumber mosaic virus (CMV; Khan and Smith, 1963), hippeastrum mosaic virus (HiMV; Brants and Van den Heuvel, 1965), hippeastrum streak virus (HiSV; Van–Velsen, 1967), tobacco mosaic virus (TMV; de–Leeuw, 1972), Amaryllis mosaic virus (AmMV; Nowicki and Derrick, 1974) and hippeastrum latent virus (HiLV; Brolman–Hupkes, 1975). In India, a mosaic disease was reported to infect 38 different varieties and cultivars of

hippeastrum (Padma and Raychaudhuri, 1976). Plants infected with HiMV showed irregularly dark and light green spots on both sides of the leaves. Besides these, there are reports of mixed infection due to two or more viruses in this genus and other related bulbous ornamentals. Therefore, studies were carried out to identify a mixed infection on *Hippeastrum stylossum* cultivated as an ornamental in nurseries in Delhi and Kalimpong in India, by poty and carlaviruses. Serodiagnostic reagents were prepared and diagnostic methods were used to detect and partially characterize the mixture of viruses.

MATERIALS AND METHODS

Collection and maintenance of virus culture

Bulbs of *Hippeastrum spp.* plants were collected from four locations *viz.* Sunder Nursery, Hauz Khas, Horticultural Field and Biochemistry Division of the Indian Agricultural Research Institute (IARI) at New Delhi and the Regional Station of IARI at Kalimpong, West Bengal, India. These bulbs were raised in the glass house in sterile soil and young leaves emerging were used as virus culture in the present studies.

Host range and insect transmission

Symptomatic leaves were macerated in liquid nitrogen and homogenised (1:1 w/v) with phosphate buffer (pH 7.2). The sap, thus extracted was rubbed on celite dusted leaves of healthy seedlings of *Allium cepa* L., *A. sativum* L., *Chenopodium album* L., C. *amaranticolor* L., *C. murale* L., *Cucumis melo* L., *C. sativus* L., *Cucurbita moschata* (Dvch.) Poir., *Datura stramonium* L., *Gomphrena globosa* L., *Hyoscyamus niger* L., *Lablab purpureus* (L.) Sweet., *Lycopersicon esculentum* Mill., *Nicotiana glutinosa* L., *N. tabacum* L. *cv.* Samsun, *N. tabacum* L. *cv.* Xanthi, *Phaseolus vulgaris* L., and *Vigna unguiculata* (L.) Walp. The leaves were rinsed with water and maintained in an insect proof glass house up to six weeks for observations. Plants showing local lesions or systemic symptoms were tested by back inoculation, leaf dip electron microscopy and DAC–ELISA. Four aphid species *viz. Aphis gossypii, Brevicoryne brassicae, Lipaphis erysimi* and *Myzus persicae* were evaluated for their efficacy as vectors. Aviruliferous apterous aphids of each species were given a preacquisition fasting of 2 h followed by an acquisition access of 30–60 sec on mosaic affected leaves.

These were then allowed to feed on healthy test plants of *N. glutinosa, N. tabacum, C. amaranticolor, H. niger* and *H. stylossum* for 24 h (10 viruliferous aphids per plant).

Virus purification

Virus was purified using a modified protocol from earlier reports (Brants and Van den Heuvel, 1965; Ramachandran *et. al.*, 1984). Infectious sap was clarified using 0.05% thioglycollic acid and 6% cold butanol followed by differential centrifugation. The dissolved pellet was passed through a 5 ml sucrose pad (30% w/v) and then subjected to gradient centrifugation in a linear sucrose column 10–40% (w/v) at 100,000 g for 90 min. The final pellet was dissolved in 200 µl of 0.05 M phosphate buffer (pH 7.2), centrifuged at low speed and the supernatant was stored in 4°C for UV absorbance, electron microscopy and serological studies.

Estimation of molecular weight of virus coat protein

The size of virus capsid protein was determined by SDS–PAGE method as described by Laemmli (1970). Test samples were mixed with sample buffer (1:1), boiled for 3 min., instantly cooled on ice, added bromophenol blue and electrophoresed in polyacrylamide gel; 12% resolving gel (10 cm) overlaid with 5% stacking gel in a vertical assembly, at 80–90 v for 1.5 to 2 h. Protein markers (Genei, Bangalore); phosphorylase–b (97.4 k), bovine serum albumin (68.0 k), ovalbumin (43.0 k) carbonic anhydrase (29.0 k) and lysozyme (14.3 k) were used for comparison. Gel was stained with 0.1% Coomassie blue (R–250) and photographed.

Production of polyclonal antiserum

A New Zealand white rabbit was immunized with freshly purified virus preparation (500 µg ml^{-1} of virus injection) emulsified with Freundt's incomplete adjuvant (1:1) intramuscularly at weekly intervals. Four such injections were administered and the rabbit was bled in two weeks after the last injection and thereafter at weekly intervals. About 15 ml blood was collected each time, allowed to coagulate at room temperature for 1 h and kept overnight at 4°C. Clear serum was decanted, centrifuged and the supernatant was mixed with an equal volume of glycerol and 0.02% sodium azide and stored at -20°C.

Serology

Serological studies were conducted to determine titre of antiserum and relationship of experimental virus with filamentous viruses using direct antigen coated enzyme linked immunosorbent assay (DAC–ELISA), immunosorbent electron microscopy (ISEM) and electro blot immuno assay (EBIA) tests. The DAC–ELISA was done in polystyrene plates using the modified protocol described by Clark and Bar Joseph (1984). The clarified antigens at 1:10 dilution were prepared by extracting fresh leaf tissue in coating carbonate buffer (1:10 w/v) containing PVP (2% w/v). The plates were first coated with 200 µl test antigens and incubated at 37°C for 1 h. After washing in PBS–T and blocking in 5% BSA, antiviral antibody in PBS–T was added in 1:1000 dilution and incubated at 37°C for 2 h. The plates were then washed with PBS–T three times (2–3 min each) and incubated in anti–rabbit immunoglobulin–alkaline phosphatase conjugate (1:6000). About 30 min after adding the substrate (0.6 mg ml^{-1} para nitrophenyl phosphate) the reaction was evaluated at 405 nm absorbance. An ISEM test was conducted as described by Milne and Lesemann (1984). For decoration of virus particles, antiserum at 1:50 dilution was used and grids were incubated for 40–45 min. For EBIA test the protein bands observed in a SDS–PAGE were transferred to nitrocellulose membrane (NCM) through electro blotting at 50 v for 2–3 h (O'Donnell *et al.*, 1982). Membranes were then transferred to a solution containing specific antibody at 1:500 dilution followed by alkaline phosphatase conjugated secondary antibodies for 1 h each. A mixture of nitroblue tetrazolium (NBT) and bromo–chloro indolyl phosphate (BCIP) was used as the substrate.

RESULTS

Symptomatology

The *Hippeastrum* plants grown from bulbs in the glass house showed mosaic symptoms consisting of irregularly scattered light and dark green regions on the lamina. With increasing plant growth, symptoms became very prominent on entire leaf. As flowers matured, yellowish white patches were seen on the scape (inflorescence stalk) which rarely merged into the normal green colour of the stalk. The colour breaking symptoms on flowers were not observed in natural varieties and hybrids. Plants raised from bulbs from different locations did not show any marked difference in symptoms. Based on symptoms it was found that the disease was widely distributed in the four locations and appeared in mild to severe form ranging from 4-92%.

Host range and insect transmission

Among the 20 species belonging to seven plant families tested, *G. globosa* produced systemic symptoms 15–20 days after inoculation. Back inoculation from systemic symptoms did not reinfect *G. globosa* or other hosts tested. Local lesions appeared on *H. niger* at 12–15 days after inoculation as small chlorotic spots, 1–2 mm in diameter with a prominent halo around. These reinfected *H. niger* only. On *C. amaranticolor*, local lesions appeared 15–20 days after inoculation as chlorotic spots 1–2 mm in diameter, coalescing within 7 days but these lesions did not reinfect *C. amaranticolor*. Four aphid species tested failed to transmit the disease from *Hippeastrum spp.* to *Hippeastrum spp.* or other herbaceous hosts.

Virus morphology

In electron microscope, leaf dip preparations from test samples showed the presence of filamentous virus particles ranging from 450–950 nm (Figure 1a), with two maxima, one at 600–650 nm and other at 750–800 nm (Figure 1b).

Virus purification

The protocol developed in the present study gave a purified virus yield of 3.5–5.5 mg virus kg^{-1} of leaf material. The absorbance profile of purified virus particles showed a maxima at 260 nm and minima at 240 nm. The ratio of absorbance (260:280) was 1.19, a value very similar to viruses belonging to poty and carla groups.

Virus coat protein

In SDS–PAGE, when purified sample was electrophoresed, it migrated as two bands with different electrophoretic mobility. Molecular weights of these bands were 27 and 33 kDa (Figure 2a, b).

Serology

Titre of the antiserum produced during the study was 1:512 in tube precipitin test and 1:1024 in ELISA and ISEM tests. In ELISA test, the

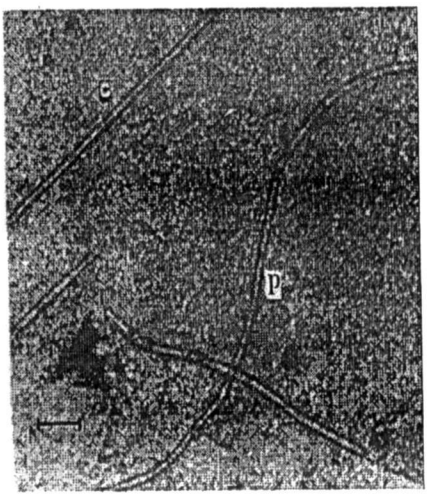

Figure 1a. Leaf dip preparation from mosaic affected *H. stylossum* leaf virus particles showing (c).

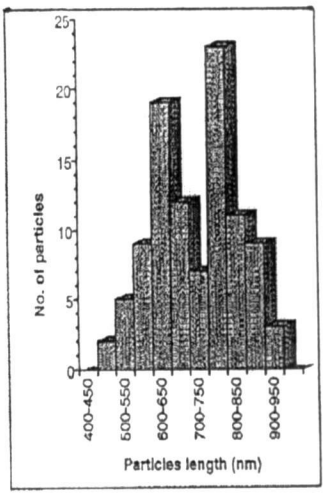

Figure 1b. Frequencydistribution of hippeastrum mosaic virus in leaf–dip preparation.

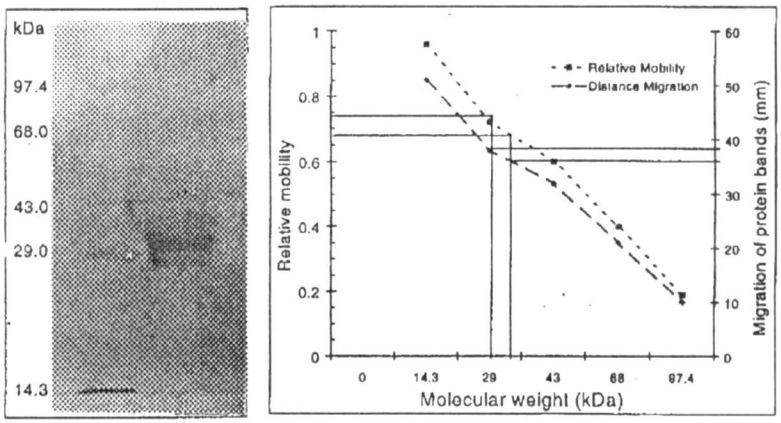

Figure 2. (a) SDS–PAGE electrophorogram of hippeastrum mosaic virus(es); coat protein; (b) Molecular weight of coat protein of hippeastrum mosaic virus(es).

polyclonal antiserum produced reacted strongly with the homologous antigen. With antigens of PVY and garlic mosaic virus–Delhi isolate (GMV–D), antiserum produced reacted mildly showing its relationship to these two virus isolates. On the other hand, the antigen under study reacted strongly with antisera to CLV, SLV (Wageningen, Netherlands), OYDV (INRA, France), PVY (ACPV, New Delhi) and mildly to GMV–D. There was no reaction with antisera to garlic latent virus (GLV), henbane mosaic virus (HMV), leek yellow stripe virus (LYSV), peanut stripe virus (PStV) and watermelon mosaic virus–1 (WMV–1).

In ISEM test too, the antigen under study decorated well with the antisera to CLV, SLV, OYDV and PVY but not to GMV–D. However, complete decoration of all the particles was achieved only when a mixture of antisera to all the four viruses were used (Figure 3).

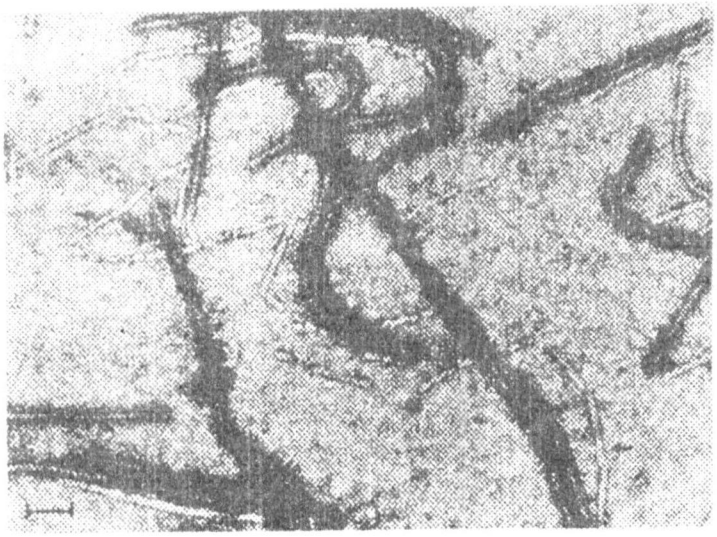

Figure 3. Virus particles decorated with PVY antisera in purified preparation (Bar. 100 nm) → Undecorated particles.

No decoration was observed with antisera to GLV, GMV–D carla viruses, PVX potex virus, bean yellow mosaic virus (BYMV), cowpea aphid

borne mosaic virus (CABMV), datura mosaic virus (DMV), eggplant mottle virus (EMoV), maize dwarf mosaic virus (MDMV), sugarcane mosaic virus (SCMV), HMV, LYSV, PStV and WMV–1 potyviruses. These results confirmed that two poty (PVY and OYDV) and two carla viruses (CLV and SLV) are associated with the disease and the homologous antiserum is a compound that has antibodies to these four viruses. The antisera of different viruses were used to decorate the antigens from four different sources. The results indicated that hippeastrum mosaic sample from Kalimpong decorated with OYDV and PVY potyvirus antisera only, whereas hippeastrum mosaic sample from the three sources in New Delhi showed decoration with four viruses.

In EBIA, purified virus preparation was transferred onto NCM and probed separately using antisera of four viruses *viz.*, CLV, SLV, OYDV and PVY. The results revealed a strong reaction with antisera to CLV, OYDV and PVY and mild reaction to SLV (Figure 4).

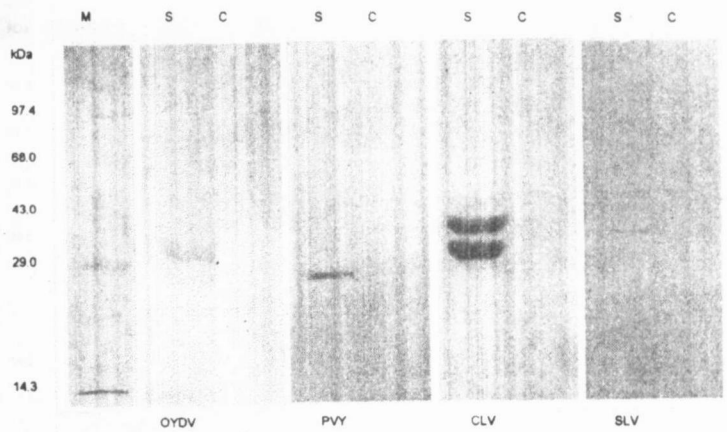

Figure 4. Serological relationship in EBIA ('M' - Marker, 'S' - sample, 'C' - Control line).

The EBIA test was used to investigate whether antisera to all four viruses are produced simultaneously. For this, virus antigens transferred to NCM was probed with homologous antisera collected from successive bleeds

(*i.e.* 1st, 2nd and 3rd) 15 days after completing the immunization schedule. When the 1st bleed antiserum was used as a probe only one band was observed on NCM indicating presence of antibodies to one virus (Figure 5b). In order to identify the virus band, the test sample was co–run with PVY on SDS–PAGE, cut into two halves, one blotted with PVY antiserum and the other with homologous antiserum from 1st bleed. The bands observed in both the halves were of similar electrophoretic mobility (Figure 5a). This indicated that the first bleed antiserum contained antibodies to PVY. Similarly, it was observed that in second bleed antiserum, antibodies were produced against more than one virus as three bands were identified. However, in 3rd bleed antibodies against all the viruses were produced as the blots clearly showed four bands of different electrophoretic mobilities (Figure 5c, d).

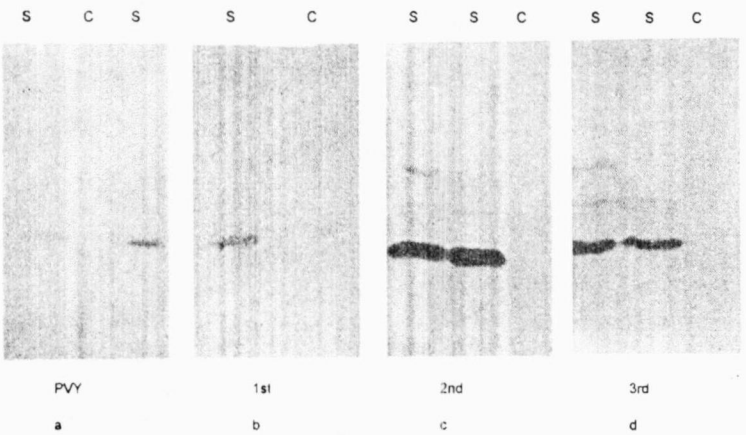

Figure 5. Antigenicity of virus(es) in successive bleeds (a) purified test antigen probed with PVY antiserum; (b-d) purified test antigen probed with homologous antiserum ('S' - sample, 'C' - control line).

DISCUSSION

Results of the present study show that hippeastrum mosaic disease on *H. stylossum* is caused by a mixture of at least four viruses *viz.* PVY and OYDV of potyvirus group and CLV and SLV of carlavirus group. Among

several viruses reported to infect *H. hybridum*, HiMV and AmMV belong to potyvirus group. Occurrence of mixed infection on *H. hybridum* due to poty and carlaviruses has been reported from Netherlands (Brolman–Hupkes, 1975). Four viruses namely, CMV, HiLV, HiMV and TMV have also been reported to cause mixed infection on hippeastrum from Lithuania (Navalinskiene *et al.*, 1993), in which HiLV (carla) and HiMV (poty) are the causal agents. The HiMV is known to cause natural infection on *Eucharis grandiflora* together with another filamentous virus similar to HiLV (Jayasinghe and Dijkstra, 1979).

In the present studies, electron microscopic observations revealed that virus particles were distributed in a range of 450–950 nm with two maxima suggesting the presence of viruses belonging to carla and potyvirus groups. Results of SDS–PAGE analysis also revealed two bands of coat protein in 27 and 33 kDa ranges. This showed that test antigen contained viruses serologically related to two carlaviruses–CLV and SLV and two potyviruses–PVY and OYDV. In ISEM tests, complete decoration of all the particles was achieved only when mixture of antisera of the four viruses, namely CLV, SLV, OYDV and PVY were used. The presence of mixture of four viruses was further evidenced by the strong reaction obtained in EBIA test (Figure 4) by probing purified antigen with antisera to *viz.* CLV, SLV, OYDV and PVY viruses. Results obtained on testing antiserum samples in successive bleeds after immunization also showed that the antiserum produced against the antigen under study is a compound one. Among the four viruses PVY is highly antigenic and could induce antibodies within fifteen days after the fourth injection. There are, to date, no reports available on the differences in antigenicity of viruses in different bleeds.

The potyvirus PVY to which the antigen under study showed positive serological reaction is nearly similar to hippeastrum mosaic virus reported in inducing local lesions on *H. niger* and length of virus particle. However, its serological relationship with HiMV type isolate is not yet studied. The other potyvirus OYDV, has only been found serologically related to the test sample, as transmission to onion mechanically or by insects could not be obtained. The carlaviruses CLV and SLV have also been found to be only serologically associated. The virus particles found in the range of 600–650 nm, lack of insect transmission and very limited host range are characteristic of presence of carlaviruses. However, in the present studies antiserum to GMV–D did not decorate any of the test antigens whereas the antigen of GMV–D isolate reacted with CLV and SLV antisera (Ghosh and Ahlawat, 1997). This shows that the carlaviruses encountered in our study are not similar to the carlavirus Delhi isolate present in garlic. Based on these results, it is concluded that *H.*

stylossum cultivars are infected by viruses serologically related to CLV and SLV carlaviruses, OYDV and PVY potyviruses. Employing the diagnostic tools developed in the present study samples of mosaic infection from Kalimpong were found to contain only the potyviruses while those from three different sources in Delhi contained all the four viruses mentioned above.

CONCLUSIONS

A mosaic disease on *Hippeastrum stylossum*, which widely distributed in nurseries in Delhi and Kalimpong, is sap transmissible. Four viruses serologically related to CLV and SLV (carlaviruses) and OYDV and PVY (potyviruses) have been shown to be associated with the mosaic disease.

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