Journal of Medicinal and Aromatic Plant Sciences 26 (2004) 279-284.

Occurrence and identification of a *Cucumber mosaic virus* isolate infecting Indian long pepper (*Piper longum*)

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Received 21st July, 2003

Abstract

A virus causing mosaic mottling in Indian long pepper (*Piper longum* L.) was isolated and identified as an isolate of *cucumber mosaic virus* (CMV) on the basis of biological, physico-chemical and serological properties. The virus isolate was easily sap transmissible to *Chenopodium album*, *Nicotiana benthamiana*, *N.glutinosa*, *N.tabacum* cv. White Burley, *Physalis floridana* indicator hosts and a few other cultivated plant species including black pepper. The virus was purified from tobacco and negatively stained purified preparations contained isometric particles of about 28 nm in diameter. The molecular weight of the viral coat protein subunits was found to be 25.6 kDa. The leaf extract from diseased *Piper longum* leaves and purified preparations showed positive reaction with polyclonal antiserum of CMV both in direct antigen coated enzyme linked immunosorbent assay (DAC-ELISA) and electro-blot immunoassay (EBIA). Particle morphology, antigenic relationships and molecular weight of coat protein suggest that the virus associated with Indian long pepper is an isolate of CMV.

Key words: Long pepper, *Cucumber mosaic virus*, occurrence, purification, host range, serology.

Introduction

Indian long pepper (*Piper longum* L.) a wild relative of black pepper occurs throughout India, Sri Lanka, Malaysia and other South East Asian countries. Long pepper fruits and roots are the most important among medicinal plants used in the Indian system of medicine such as Ayurveda, Unani and Sidha. It is grown wild in Assam, West Bengal, Bihar, North Eastern regions, Uttar Pradesh, Kerala, Tamil Nadu and Andhra Pradesh. It is also cultivated on a small scale in Tamil Nadu, Andhra Pradesh, Assam and West Bengal [9]. A mosaic disease on long pepper was noticed at Calicut during 1985 and recently noticed in Nellore district of Andhra Pradesh where long pepper is grown in oil palm plantations as an intercrop. The disease was characterized by mild mosaic on leaves but in severe cases, crinkling of leaves with severe mosaic and dark green blisters on leaves along with severe stunting of infected vines was noticed [11]. The disease could be transmitted through mechanically onto indicator hosts indicating viral nature of the disease. This paper reports the identification of the causal virus as an isolate of cucumber mosaic virus (CMV) based on host range, particle morphology and serological studies.

Materials and methods

Virus isolate

Symptomatic long pepper leaves showing mosaic were used as source of inoculum in the experiment. Indicator hosts namely *Chenopodium album*, *Nicotiana benthamiana, N.glutinosa, N. tabacum* cv. White Burley and *Physalis floridana* were used to isolate the virus by mechanical inoculation from long pepper.

Mechanical inoculation

Mechanical inoculations were done by extracting sap in chilled 0.1M phosphate buffer (pH 7.2) containing 0.1% 2 mercaptoethanol in a mortar kept in an ice tray. The extracted sap was rubbed on the leaves of test plants pre-dusted with celite or carborandum powder.

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Host range

The following plants belonging to seven plant families were grown in pots raised under insect proof glass house and were rub-inoculated: *Cajanus cajan*, *Capsicum annuum*, *Chenopodium album*, *Citrullus lanatus*, *Cucurbita pepo*, *Cucumis sativus*, *Glycine max*, *Gossypium* sp., *Helianthus annuus*, *Luffa acutangula*, *Momordica charantia*, *N. benthamiana*, *N. glutinosa*, *N. tabacum*, *Phaseolus vulgaris*, *Physalis floridana*, *Piper nigrum* cv. Karimunda, *Vigna mungo*, *V. radiata*, and *V. unguiculata*. A minimum of five plants of each species were inoculated and kept under observation for four weeks. Inoculated plants were checked for the virus presence by back inoculation onto tobacco (*N.benthamiana*)

Virus purification

Virus isolated from naturally infected long pepper was propagated in *N. tabacum* cv. White Burley and *N. benthamiana*, which were used as the source for virus purification. Purification was carried out using the procedure [7] with some modification. Young infected leaves collected 2-3 weeks after inoculation were triturated at 1:2 dilution (w/v) in 0.5M sodium acetate buffer pH 6.4 containing 5 mM ethylene diamine tetra acetic acid (EDTA) di-sodium salt and 0.5% thioglycolic acid. The sap was expressed through muslin cloth and emulsified with an equal volume of chloroform by stirring for 30 minutes at 4°C followed by centrifugation at 12,000g for 10 minutes. The aqueous layer was removed and the virus was precipitated by adding polyethylene glycol (PEG 6000, 10%) in the presence of sodium chloride (1.75%, w/v) with continuous stirring for 1 hour. The virus was sedimented by centrifugation at 12,000g for 15 minutes. The resulting pellet was suspended overnight in one-fifth original volume of 5 mM sodium borate buffer, 0.5 mM EDTA, pH 9.0. Triton X 100 was added to a final concentration of 2% (v/v) in the suspension, stirred for 30 minutes and centrifuged at 18,000g for 25 minutes. The supernatant obtained was subjected to centrifugation at 45,000g for 3 hour in an ultracentrifuge (Beckman Optima L 80K). The pellet was dissolved in about 3 ml of 5 mM borate buffer containing 0.5mM EDTA, pH 9.0. Further purification was carried out by layering partially purified preparation on a linear 10-40% (w/v) preformed sucrose density gradient (prepared by layering 5 ml of 10% and 10 ml each of 20%, 30% and 40% sucrose in 5 mM borate buffer containing 0.5mM EDTA, pH 9.0 and incubating overnight at 4°C). Gradients were centrifuged at 55,000g for 2 hours using SW 28 rotor (Beckman). The virus containing band was located with the help of light vertically passing through the tube. The band was collected, dialysed and concentrated by centrifugation at 45,000g for 3 hours. A 260/280 ratio of the virus preparation was measured using spectrophotometer (Carry 50 Bio-spectrophotometer, Australia). The virus yield was determined by considering A_{260} 5.0=1 mg/ml [10].

Electron microscopy

Electron microscopy of leaf dip and purified preparations of the virus negatively stained with 2% uranyl acetate (pH 4.5) were examined under JEOL-100-CF-II transmission electron microscope at the Unit of Virology, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi 110 012.

Determination of molecular weight of coat protein

The molecular weight of the virus coat protein was determined through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% resolving gel and 5% stacking gel using the method described earlier [6], samples were mixed with an equal volume of the Laemmli sample buffer [6] heated for 3 minutes in boiling water was used for loading on the gel [6]. The marker protein (medium range; Genei, Bangalore) was used as size standard.

Serological assays

Direct antigen coated enzyme linked immunosorbent assay (DAC-ELISA) and electroblot immunoassay (EBIA) were performed to determine the reactivity of the present virus with other viruses.

DAC-ELISA was carried out as described previously [2] using antisera to cucumo-, ilar, poty- and tospo- viruses. Polyclonal antiserum to a potyvirus (*Potato virus Y*, PVY), ilarvirus (*Tobacco streak virus*, TSV) and tospovirus (*Groundnut bud necrosis virus*, GBNV) were obtained from Unit of Virology, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi. Antiserum to *Cucumber mosaic virus* (CMV) was obtained from Dr S.D. Yeh, Chung Hsing National University, Taiwan. Sap extract was

prepared by grinding leaf tissue in 10 volumes (w/v) of coating buffer containing 2% polyvinyl pyrrolidone followed by centrifugation at 3000g for 3 minutes. The supernatant was used in ELISA plate (polystyrene plates; Corning, New York, USA). Antisera were used at 1:1000 dilutions except for CMV which was used at 1:4000 while anti-rabbit IgG alkaline phosphatse conjugate (Sigma Chemical Co., St. Luis, USA) was used at 1:20,000 dilution. The reactions were read at 405 nm, 1 hour after adding the substrate (p-nitrophenyl phosphate) using an ELISA reader (μ Quant, Bio-Tek Instruments Inc., USA).

EBIA was performed after SDS-PAGE as described earlier [8] using nitrocellulose membrane and alkaline phosphatase conjugated anti-rabbit antibodies (Sigma Chemical Co., St. Luis, USA). After electroblotting at 200 V for 2-4 hours, the membranes were processed as described earlier [1]. Antiserum and alkaline phosphatase labeled antirabbit IgG were used at 1:4,000 and 1:20,000 dilutions, respectively. Pre-stained marker proteins (Bio-Rad, Richmond, CA, USA) were used as size standards.

Results and discussion

Mechanical inoculation and host range

The infected *P.longum* plants showed symptoms like crinkling, mosaic and dark green blisters on leaves (Figure-1) Leaves become leathery or brittle and small in size. Infected vine showed reduced internodal length and general stunting of vines. The causal virus was efficiently sap transmitted to the indicator hosts (*C. album*, *N. benthamiana*, *N. glutinosa*, *N. tabacum* cv. White Burley and *P. floridana*) tested. Chlorotic local lesions appeared on *C. album* within 3-4 days after inoculation (Figure-2A). The symptoms appeared 7-10 days after inoculation on other hosts. Symptoms on the *N. benthamiana* included severe puckering, mosaic mottling and downward curling of leaves (Figure-2B) while in *N. glutinosa*, vein clearing followed by mosaic and yellow mottling with slight curling at leaf margins were the prominent symptoms (Figure-2C). Sap inoculated *N. tabacum* cv. White Burley and *P. floridana* plants showed dark green mosaic followed by dark green blister like symptoms on the leaves (Figures-2D & 2E). The virus could be back inoculated from these hosts onto *P. longum*, which developed mild mosaic symptoms 3 weeks after inoculation similar to those observed in the field conditions. When *P. nigrum* seedlings at four-leaf stage were inoculated, mosaic followed by narrowing of leaves and severe stunting of plants were

Figure- 1: Naturally infected *P.longum* plant showing mosaic, crinkling, leathery leaves along with stunting of plant.

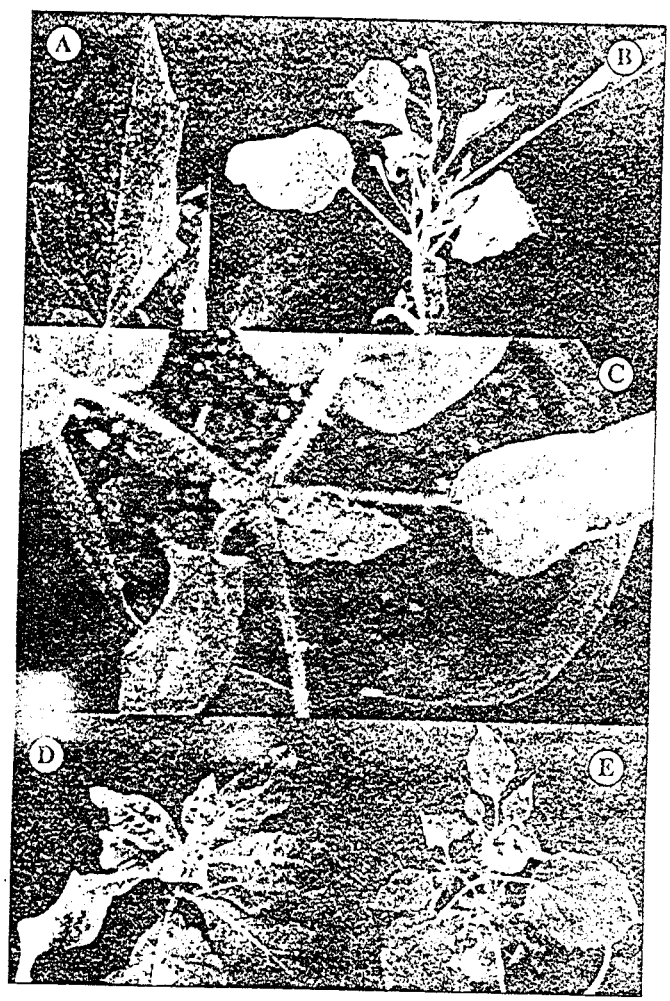

Figure- 2: Sap inoculated *Chenopodium album* (2A), *Nicotiana benthamiana* (2B), *N.glutinosa* (2C), *N.tabacum* cv White Burley (2D) and *Physalis floridana* (2E) plants showing various kinds of symptoms

seen. Besides the virus was also transmitted to other cultivated hosts such as *C. sativurs* and *C. annuum*, which showed systemic, mosaic symptoms. Back inoculation to *N. benthamiana* from test plants showed that only susceptible hosts reproduced the symptoms on *N. benthamiana*..

Virus purification

The virus was purified from *N. benthamiana* and *N. tabacum* cv. White Burley, as they were good propagating hosts. The purification procedure resulted in a clean virus preparation as judged by electron microscopy and spectrophotometry. The A 260/280 ratio of the purified preparation was 1.56 and yield of virus obtained per 100g of tissue varied from 2-5 mg depending on the harvest time after inoculation.

Electron microscopy

Electron microscopic studies of purified preparations revealed the presence of isometric particles measuring about 28 nm in diameter resembling to CMV (Figure-3). Similar kind of particles was seen in leaf

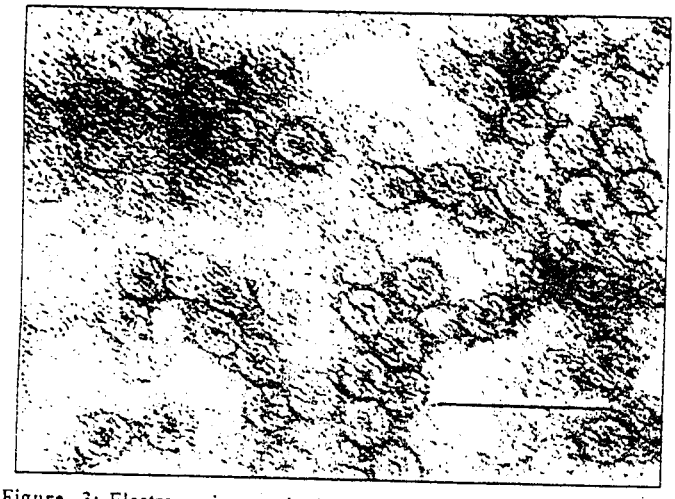

Figure- 3: Electron micrograph showing purified preparations of the virus. Bar represents 100 nm.

dip preparations from sap inoculated indicator hosts but not in the naturally infected *P.longum* leaves. This may by due to the low concentration of virus particles in naturally infected *P.longum* plants.

Molecular weight of coat protein

The coat protein of disrupted virions in SDS-PAGE was resolved into one species with a molecular weight of 25.6 kDa (Figure-4) which is close to the value reported for black pepper isolate of CMV [10].

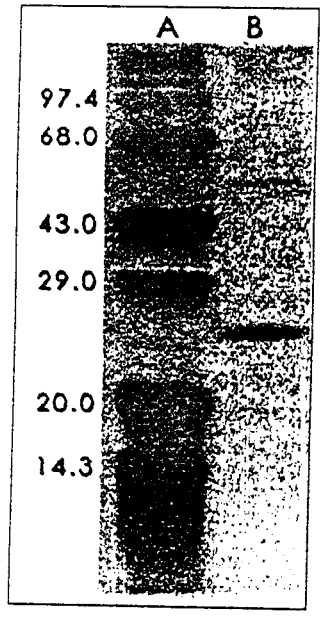

Figure- 4: SDS-PAGE analysis of coat protein of the purified virus. Lane A: Marker protein and numbers on left indicate MW of marker proteins; Lane B: purified virus from *P. longum*.

Serological assays

In DAC-ELISA, infected long pepper samples reacted with only CMV antiserum suggesting the association of a CMV with diseased samples (Table-1). No reaction was observed with antisera to other viruses used suggesting the lack of association of an ilar-, poty- or tospo- virus with the disease. EBIA test also gave positive reaction with CMV antiserum further confirming that virus is antigenically related to CMV (Figure-5).

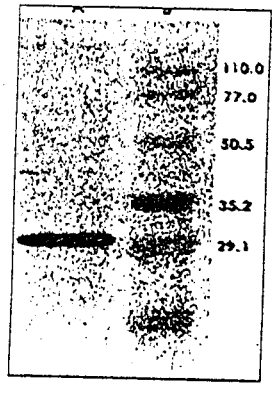

Figure- 5: Electro-blot immunoassay (EBIA) analysis of coat protein of the virus associated with diseased *P. longum*. Lane A: purified virus; Lane B: Bio-Rad pre-stained protein standards and numbers on right indicate MW of marker proteins. Blotted virus protein was probed with antiserum of CMV.

Our results clearly indicate that mosaic disease on Indian long pepper (*P.longum*) has a viral etiology. The information presented on limited host range, particle morphology and antigenic relationship studies through DAC-ELISA and EBIA suggest that the virus is an isolate of CMV. Natural infection of CMV is already known in black pepper (*P.nigrum*). from Brazil, India and Sri Lanka [3, 4, 5, 10]. The successful sap transmission of CMV from *P. longum* to *P. nigrum* indicates the closeness of CMV isolates infecting both these species. This data is further supported by host range studies of CMV infecting *P. longum* and *P. nigrum*. CMV infecting black pepper also has a similar host range [3, 10]. Further the A260/280 ratio and coat protein molecular weight of CMV infecting *P. longum* is also close to the values reported for CMV infecting *P. nigrum* [3, 10]. To unequivocally identify whether CMV isolates infecting black pepper and Indian long pepper are same or distinct ones, the virus will have to be further characterized at molecular levels. As it has been shown that infected long pepper could become a source of infection to black pepper, care should be taken to eliminate infected long pepper vines especially near the black pepper plantations. As both long pepper and black pepper are clonally propagated, disease spread is rapid through planting material when infected plants are used as source of planting material. As symptoms alone cannot be used as criterion for confirming the disease-free nature of the materials, it is necessary to develop methods to quick, reliable and sensitive detection (either serological or nucleic acid based) of the virus for producing and certifying virus-free planting material. This is the first report of characterization of CMV infecting *P.longum*.

Table-1 Reaction of diseased *P.longum* and *P.nigrum* samples to *Cucumber mosaic virus* (CMV) antiserum in direct antigen coated (DAC)-ELISA*

Isolate	A 405 value #
P. longum	
Sample 1	1.40
Sample 2	1.11
Sample 3	0.87
Sample 4	1.56
P.nigrum	
Sample 1	0.13
Sample 2	0.42
Sample 3	0.53
Sample 4	0.43
Nicotiana benthamiana$	3.10
N. glutinosa$	2.53
N.tabacum cv. White Burley$	2.34
Physalis floridana$	2.12
Healthy *P.longum*	0.06
Helathy *P.nigrum*	0.04
Healthy *N. benthamiana*	0.03

* antigen and antiserum were used at 1:10 and 1:4000 dilutions respectively

#Average of three replications taken 1 h after addition of substrate

$Artificially inoculated through sap under green house

Acknowledgements

We are thankful to Scientist in charge, Unit of Virology, Indian Agricultural Research Institute, New Delhi for electron microscopy studies and to Dr S.D.Yeh, Chung Hsing National Univsersity, Taiwan for providing antiserum to CMV.

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