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Purification, production of antiserum and development of enzyme linked immunosorbent assay-based diagnosis for *Cucumber mosaic virus* infecting black pepper (*Piper nigrum* L.)[1]

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Abstract

Cucumber mosaic virus (CMV) infecting black pepper (*Piper nigrum*) in India was propagated on *Nicotiana benthamiana* and *N. glutinosa*, and purified by differential and sucrose density-gradient centrifugation. The purified virus preparations showed the presence of typical isometric particles of about 28 nm diameter. Polyclonal antiserum against the virus was produced in New Zealand white rabbits. Immunoglobulin G was purified from the crude antiserum and coupled with the enzyme, alkaline phosphatase. 'Double antibody sandwich-enzyme linked immunosorbent assay method was standardized for detection of CMV in diseased black pepper samples collected from different regions of Karnataka, Kerala and Tamil Nadu. The virus infection was also detected on other *Piper* species such as *P. chaba*, *P. colubrinum* and *P. longum* and a few of the common weeds such as *Ageratum conyzoides*, *Colacasia esculanta*, *Synedrella nodiflora*, *Cynodon dactylon* and *Sonchus oleraceus* found in and around black pepper gardens. The utility of the method developed in diagnosis, epidemiology and management of the disease is discussed.

Key words: black pepper, *Cucumber mosaic virus*, detection, double antibody sandwich-enzyme linked immunosorbent assay, *Piper nigrum*.

Introduction

Among the biotic stresses affecting black pepper (*Piper nigrum* L.) in India, infection by viruses is becoming increasingly important due to their widespread occurrence and yield loss caused by them. Viruses belonging to the genera *Badna* and *Cucumo* have been reported to be associated with stunted disease of black pepper in India (Sarma *et al.* 2001; Bhat *et al.* 2003). The disease caused by *Cucumber mosaic virus* (CMV) is characterized by small, crinkled, brittle, leathery leaves and chlorotic patches/streaks on leaves. In severe cases, the leaves become abnormally narrow with reduced internodal length, leading to typical stunting of plants (Fig. 1). The disease caused by *badnavirus* is characterized by chlorotic mottling, chlorosis, vein clearing, leaf distortion, reduced plant vigour and poor fruit set. In

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most cases mixed infections with both the viruses are seen. The disease is becoming important with 100% incidence in certain black pepper gardens (in many of the local cultivars and Karimunda) especially in Idukki and Wayanad districts of Kerala. CMV infection on black pepper has also been reported from Brazil (Duarte *et al.* 2001) and Sri Lanka (De Silva *et al.* 2001; 2002). The virus is transmitted from one generation to other through the use of infected stem cuttings of black pepper (De Silva *et al.* 2001; Sarma *et al.* 2001; Bhat *et al.* 2003).

Lack of a proper detection methods has led widespread distribution of these viruses in black pepper growing regions of the country through infected asymptomatic planting material. Hence, planting of virus-free cuttings is very important to check further spread of the virus. In order to check the virus-free nature of the planting material, use of sensitive and reliable diagnostic methods are essential. Enzyme linked immunosorbent assay (ELISA) based serological assays are the most reliable methods for identifying viruses and the disease caused by them. Presently, the only method available for diagnosis of the disease is symptomatology. This may not be reliable as the symptoms are highly variable and depending on the season, growth stage and other factors, the disease can be difficult to identify or detect visually. This paper describes the isolation of CMV infecting black pepper plants, production of polyclonal antiserum against the virus and development of an ELISA based methodology for detecting the virus in diseased plants.

Materials and methods

Virus isolate

CMV affected black pepper plants (identified by both symptoms and ELISA using heterologous CMV antiserum) were collected from Kodlipet (Kodagu District, Karnataka) and the virus was maintained and propagated on *Nicotiana benthamiana* W. and *N. glutinosa* L. by mechanical inoculation. Mechanical inoculation was carried out by extracting sap in chilled 0.1 M phosphate buffer (pH 7.2)

Fig. 1. *Cucumber mosaic virus* infected black pepper plant

containing 2-mercaptoethanol (0.1%) in a mortar kept in an ice tray. The extracted sap was rubbed on the leaves of test plants dusted with celite or carborandum powder.

Virus purification

Virus isolated from naturally infected black pepper plants was propagated on *N. benthamiana* and *N. glutinosa*, which were used as the source for virus purification. Purification was carried out using the procedure of Lot *et al.* (1972) with some modifications. Young infected leaves collected 2 to 3 weeks after inoculation were ground at 1:2 dilution (w/v) in 0.5 M sodium acetate buffer (pH 6.4) containing 5 mM ethylenediaminetetraaceticacid (EDTA) di-sodium salt and 0.5% thioglycolic acid. The sap was passed through muslin cloth and emulsified with an equal volume of chloroform by stirring for 30 min at 4°C followed by centrifugation at 12,000 g for 10 min. The buffer layer was removed and the virus was precipitated by adding polyethylene glycol (PEG 6000, 10%, w/v) in the presence of sodium chloride (1.75%, w/v) with continuous stirring for 1 h to precipitate the virus. The virus was sedimented by centrifugation at 12,000 g for 15 min. The resulting pellet was suspended overnight in one-fifth original volume of 5 mM sodium borate buffer containing 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 min and centrifuged at 18,000 g for 25 min. The supernatant obtained was subjected to centrifugation at 45,000 g for 3 h in an ultracentrifuge (Beckman Optima LE 80K). The pellet was dissolved in about 3 ml of 5 mM borate buffer containing 0.5 mM EDTA, pH 9. 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 0.5 mM EDTA, pH 9 and incubating overnight at 4°C). Gradients were centrifuged at 55,000 g for 2 h 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 45,000 g for 3 h. A 260/280 ratio of the virus preparation was measured using spectrophotometer (Carry 50 Bio-spectrophotometer, Australia).

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.

Antiserum production

The polyclonal antiserum against the whole virus was prepared in New Zealand white rabbits by injecting purified virus preparations intramuscularly six times at 10 day intervals. On each occasion, 500 µl purified virus containing 0.5 mg of virus emulsified with incomplete Freund's adjuvant (1:1, v/v) was injected. The animal was bled 15 days after the last injection and antiserum collected.

Purification of IgG and preparation of enzyme conjugate

Immunoglobulin G (IgG) was purified from the crude polyclonal antiserum by affinity chromatography. Affinity column contained protein A coupled to cyanogen bromide activated agarose (Genei, Bangalore). Five ml of polyclonal antiserum was passed through the column and the column was washed with 25 ml of wash buffer to remove all unbound materials. The IgG bound to the column was later eluted by adding 5 ml of elution buffer and quantified by taking OD values at 280 nm (1.4 OD=1 mg ml^{-1} of IgG). One mg of this IgG was used for conjugate preparation. One step glutaraldehyde method described by Avrameas (1969) was followed for the preparation of IgG-alkaline phosphatase conjugate.

Double antibody sandwich (DAS) ELISA

DAS-ELISA was done on polystyrene plate (Co-Star) using the protocol described by Clark *et al.* (1986). Wells were initially coated with CMV IgG at 1µg ml^{-1} in coating buffer. Antigen preparation included grinding leaf tissues in five volumes of PBS-T containing 2% polyvinyl pyrrolidone (PVP) and 0.2% BSA followed by centrifugation at 8000 rpm for 1 min. Supernatant obtained was used to load onto ELISA plates. CMV specific alkaline phosphatase conjugate was used at 1:500 dilution. The reactions of ELISA were read at 405 nm, 1 h after addition of substrate (p-nitrophenyl phosphate, Genei, Bangalore) by using an ELISA reader (µQuant, Bio Tek Instruments Inc., USA).

Results and discussion

The CMV infecting black pepper was mechanically transmitted and propagated on the tobacco species, *N. benthamiana* and *N. glutinosa*. Symptoms appeared within 7–10 days in these inoculated plants. Symptoms on *N. benthamiana* included severe puckering, mosaic, mottling and downward curling of leaves while in *N. glutinosa*, vein clearing followed by mosaic and yellow mottling with slight curling at leaf margins were the prominent symptoms. CMV infecting black pepper in Sri Lanka was also reported to be mechanically transmitted onto *N. benthamiana* and *N. glutinosa* (De Silva *et al.* 2001). The purification procedure resulted in a clean virus preparation. A single opalescent band was seen in the sucrose density gradient centrifugation at about 3.7 cm from the top of the gradient tube. A 260/280 ratio of the purified virus

was 1.65 and yield of virus obtained per 100 g of tissue varied from 1 to 4 mg depending on the harvest time after inoculation. Electron microscopy of negatively stained purified preparations revealed the presence of isometric particles of about 28 nm diameter (Fig. 2).

The virus was immunogenic and produced good titred (1:8,000) antiserum in rabbits. Initial experiments using various concentrations of coating antibody (IgG) and conjugate with an aim to standardize DAS-ELISA was achieved at 1µg ml^{-1} of IgG coating and 1:500 dilutions of CMV IgG-alkaline phosphatase conjugate. The DAS-ELISA procedure thus standardized detected CMV in dilutions of extracts from diseased black pepper plants from different regions of Karnataka, Kerala and Tamil Nadu (Table 1). The varying OD values seen with diseased black pepper samples from different regions indicate varying virus concentration in these samples. Further, within the diseased black pepper vine, the distribution of CMV was highly variable although the virus was found in all plant parts including stems and roots.

CMV infection was also detected from other naturally infected *Piper* species such as *P. chaba* Hunter, *P. colubrinum* Link and *P. longum* L. (Table 1). Of the 79 individual *P. colubrinum* plants tested, 13 were found positive for CMV infection. *P. chaba* and *P. longum* are two important species of *Piper* cultivated largely for medicinal use while *P. colubrinum* is used as root stock to derive *Phytophthora* resistant black pepper plants. Natural infection of CMV with all these three species indicates that they may act as potential source of inoculum for cultivated black pepper. While infected *P. chaba* and *P. longum* showed mosaic and stunting symptoms, no such symptoms were seen on *P. colubrinum* infected with CMV. Thus *P. colubrinum* might act as a symptomless carrier for CMV. Higher OD values obtained with *P. colubrinum* indicate the higher virus titre in these plants compared to diseased black pepper plants. In addition, some of the common weed hosts found in and around black pepper gardens such as *Ageratum conyzoides* L., *Colacasia esculanta* (L.) Schott, *Synedrella nodiflora* (L.) Gaertn., *Cynodon dactylon* (L.) Pers and *Sonchus oleraceus* L. also tested positive for CMV infection in DAS-ELISA. CMV infection was also recorded on *Gliricidia sepium* (Jacq.) Kunth ex. Walp, one of the commonly used standards for raising black pepper plantations. Higher OD values obtained with many of the weed hosts indicate that they could act as a potential source of virus inoculum for black pepper.

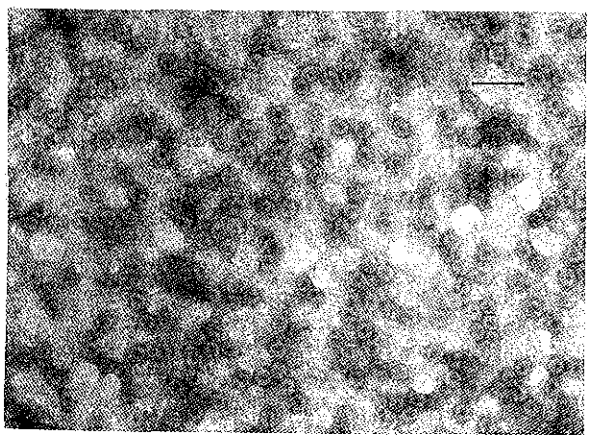

Fig. 2. Electron micrograph of purified preparations of *Cucumber mosaic virus* (bar represents 100 nm).

The DAS-ELISA based serological method used in the present study could detect CMV in diseased samples. None of the healthy black pepper plants showed positive reaction in the test thus indicating specificity of the method developed. Of the different forms of ELISA, DAS-ELISA was found to be more sensitive and highly suitable for the specific detection of plant viruses (Hull 2002). So far, detection of CMV in black pepper samples was reported using heterologus polyclonal antisera raised against different strains of CMV such as CMV-A, CMV-B, CMV-C, CMV-L, CMV-Fiji, CMV-Passiflora and CMV-T. A wide range of variation was observed in these antisera in their ability to detect CMV infection in a given diseased black pepper sample. A few of the antisera even failed to detect CMV infection in black pepper (De Silva *et al.* 2001; Sarma *et al.* 2001). Thus development of homologous antiserum against black pepper isolate of CMV in the present study will

help in the detection of CMV in all the diseased black pepper samples. This is the first report of the production of polyclonal antiserum against black pepper isolate of CMV and development of DAS-ELISA based method for the specific detection of virus in black pepper samples. The method can be successfully used to detect CMV infections in black pepper plants, cuttings and seedlings. This could also be used in commercial nurseries to detect the presence of CMV infection in stock (mother) plants and in preventing movement of infected plants to new areas. The methodology developed will help in mapping the disease, monitoring its ecological spread and identifying black pepper grow-

Table 1. Detection of *Cucumber mosaic virus* by Double Antibody Sandwich-ELISA* in *Piper* species from different regions

Piper nigrum isolate	Visual symptoms	A_{405} value$	*Piper* spp. isolate	Visual symptoms	A_{405} value$
Karnataka State			*P. chaba*		
Hassan District			Sample 1	MM, Y	0.33
Bantanahalli	M, LD, S	0.43	Sample 2	MM, Y	0.31
Belur	M, S	0.57	Sample 3	MM, Y	0.30
			Sample 4	MM, Y	0.21
Kodagu District			Sample 5	MM, Y	0.20
Balale	M	0.32			
Kodlipet	M, LD, S	0.78	*P. colubrinum*		
Mayamudi	M, S	0.37	Sample 1	-	3.92
Polibetta	M, S	0.41	Sample 2	-	3.89
			Sample 3	-	3.98
Udupi District			Sample 4	-	3.76
Idu	M, LD, S	0.68	Sample 5	-	2.00
			Sample 6	-	0.76
Kerala State			Sample 7	-	0.71
Idukki District			Sample 8	-	0.57
Adimali	M	0.75	Sample 9	-	0.51
Chakkuppalam	M, LD, S	0.22	Sample 10	-	0.35
Chottupara	M, LD, S	0.19	Sample 11	-	0.33
Muttom	M, S	0.15	Sample 12	-	0.30
Thookupalam	M, S	0.19	Sample 13	-	0.21
Vandiperiyar	M, S	0.15			
			P. longum		
Wayanad District			Sample 1	M, LD, S, B	0.53
Kenichira	M	0.49	Sample 2	M, S, B	0.46
Kuppadi	M, S	0.57	Sample 3	M, LD, S,	1.33
Pulpally	M, LD, S	2.01	Sample 4	M, LD, S, B	1.65
Vengapalli	M, S	0.78	Sample 5	M, LD, S, B	1.16
			Sample 6	M	0.22
Kozhikode District			Sample 7	M, LD, S, B	2.23
Peruvannamuzhi	M, LD, S	0.61	Sample 8	M, LD, S, B	1.03
			Sample 9	M, LD, S, B	1.98
Tamil Nadu State			Sample 10	M, LD, S, B	1.56
Coimbatore District			Sample 11	M, LD, S, B	0.99
Walayar	M	1.16	Sample 12	M, LD, S, B	1.98
Healthy black pepper	-	0.00	Healthy black pepper	-	0.04

B= blisters on leaves; LD= leaf distortion; M= mosaic; MM= mild mosaic; S= stunting of the plant; Y= yellowing; -= no visual symptoms

*Antigen and conjugate were used at 1:5 and 1:500 dilutions, respectively

$ Average of three replications, 1 h after addition of substrate

ing areas free from CMV infection. The method could also be successfully employed in the specific detection of CMV in alternate crop and weed hosts, and to identify resistance sources against CMV in black pepper.

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