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Development of ELISA-based technique for the detection of a putative badnavirus infecting black pepper (*Piper nigrum* L.)

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ABSTRACT: Bacilliform virus particles were purified from infected black pepper samples collected from the Indian Institute of Spices Research Farm at Peruvannamuzhi, Kozhikode, showing typical symptoms of stunting disease. The purified virus preparation was used to raise polyclonal antiserum in rabbit from which Immunoglobulin G (IgG) was purified and conjugated with alkaline phosphatase enzyme. This conjugate was used for detecting the presence of virus infection in black pepper samples collected from different regions of Andhra Pradesh, Karnataka, Kerala, Tamil Nadu and West Bengal by Double Antibody Sandwich (DAS) ELISA method. Samples from different *Piper* species were also tested for the presence of badnavirus infection. Occurrence of a putative badnavirus on *Piper colubrinum, P. hapnium, P. longum* and *P. mullesua* is the first report from India and elsewhere.

Key words: Badnavirus, black pepper, virus purification, electron microscopy DAS-ELISA, detection

Black pepper is an important spice condiment obtained from the dried berries of *Piper nigrum.* It has a great commercial value fetching around 88 crore rupees annually through export. India is the leading producer of black pepper in the world with the crop grown in an area of 1.92 lac hectares with 30.23-lac ton production annually (Selvan, 2002). In India the crop is grown mainly in the states of Karnataka and Kerala. Black pepper plantations face threat from diseases caused by nematodes, fungi and viruses. The first viral disease reported on black pepper was the stunting disease from Idukki district of Kerala during 1975 (Pailey *et al.,* 1981). The symptoms of the disease include mosaic, mottling, small leaf condition and stunting of the whole vine (Fig.1). The infected vines produce short spikes with poor filling, leading to the yield reduction (Holliday, 1959; Pailey *et al.,* 1981; Lockhart *et al.,* 1997; Sarma *et al.,* 2001; de Silva *et al.,* 2002). Depending on the season, growth stage and other factors, the symptoms vary from prominent to very mild. Hence symptomatology cannot be taken as the sole criteria to identify the infected plants in the field. Association of *Cucumber mosaic virus* (CMV) (Sarma *et al.,* 2001) and badnavirus serologically related to *Banana streak virus* (BSV) (Bhat *et al.,* 2003) with stunting disease of black pepper have been reported in India. Similar viruses have also been reported as the cause of viral disease of black pepper in Brazil and Sri Lanka (Duarte *et al.,* 2001; de Silva *et al.,* 2002). However, in Southeast Asian countries like

Fig. 1. Lateral branch showing bright yellow along the veins and curling of leaves.

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Malaysia, Philippines and Thailand, the disease was reported to be caused by a badnavirus only and the association of CMV has not been reported so far (Lockhart *et al.,* 1997). The badnavirus infecting black pepper in Southeast Asian countries has been named as *Piper yellow mottle virus* (PYMV). However the exact identification of the badnavirus infecting black pepper in India is yet to be determined.

The major means of spread of the viruses is through the use of infected asymptomatic cuttings. Planting of virus-free cuttings is important to check the spread of the virus. Since symptoms alone cannot be used as reliable criteria to identify virus-free planting material, use of sensitive diagnostic methods based on Enzyme Linked Immunosorbent Assay (ELISA) or Polymerase Chain Reaction (PCR) are essential. A Double Antibody Sandwich (DAS) ELISA-based method has already been reported for the detection of CMV infecting black pepper (Bhat *et al.*, 2004). This paper describes about the efforts made to detect the badnavirus infection in planting material through DAS-ELISA.

MATERIALS AND METHODS

Badnavirus infected black pepper plants exhibiting typical symptoms like chlorotic mottles, chlorotic flecks and mottling along the veins, were collected from Indian Institute of Spices Research farm, Peruvannamuzhi, Kozhikode. Initially the samples were subjected to Direct Antigen Coating (DAC) ELISA using antiserum against CMV and BSV. The samples that reacted positively to BSV antiserum and negative towards CMV, were used for the purification of the virus. The other virus isolates collected from different regions are listed in Table 1.

Virus purification

The virus was purified using the protocol of de Silva *et al.,* (2002), with few modifications. The infected leaves with distinct symptoms were homogenized with extraction buffer in 1:10 (leaf: buffer) ratio. The extraction buffer included 0.25 M Tris-HCl pH 7.4, containing 0.5% (w/v) sodium sulphite, 4% (w/v) polyvinyl pyrrolidone (PVP), 0.5% (v/v) β-mercapto ethanol and 0.25% (w/v) diethyl dithiocarbamic acid (DIECA). After grinding, the extracts were filtered through muslin and to the

Table 1. Detection of badnavirus by Double Antibody Sandwich (DAS) ELISA in different *Piper nigrum* isolates and *Piper* species.

Piper nigrum isolates	Visual symptoms	A_{405} value	*Piper nigrum* isolates	Visual symptoms	A_{405} value
KARNATAKA STATE					
Kodagu			*Peruvnnamuzhi*		
Balale	M, CF	0.26	*(contd.,)*		
Kodlipet	M, VC, S	0.61	Sample 6	M, CF	0.26
KERALA STATE			Sample 7	M, CF, VC	0.32
Idukki			**Wyanad**		
Adimali	M, C, S	0.18	*Chainthkunnu*	Asymptomatic	0.20
Thookkupalam	M, VC, S	0.40			
Kannur			WEST BENGAL STATE		
Taliparamba	M, Mo	0.39	*Shantiniketan*	M, VC	0.34
Kasargod					
Vellari kundu	M, CF	0.20	***Piper spp.***		
Kozhikode			*P. colubrinum*	M, Mo, LD	0.35
Chelavoor			*P. hapnium*	M, CF	0.26
Sample 1	M, CF	0.19	*P. longum*	M, Mo, LD	0.40
Chembanoda			*P. mullesua*	M, Mo, LD	0.28
Sample 1	M, CF	0.26			
Peruvannamuzhi			**Healthy sample**	Asymptomatic	0.14
Sample 1	M, CF	0.23			
Sample 2	M, CF	0.24			
Sample 3	M, CF	0.20			
Sample 4	M, CF	0.25			
Sample 5	M, CF	0.21			

C: chlorosis; CF: chlorotic flecks; LD: leaf distortion; M: mottle; Mo: mosaic; S: stunting of the plant.

filtrate, Triton X–100 (20 ml. L^{-1} conc.) was added and stirred for a period of 35 min at room temperature. The extract was centrifuged at 12000 g for 20 min at 10ºC and supernatant was collected. To this supernatant 4% polyethylene glycol (PEG-6000) and 0.2 M NaCl were added and stirred for 4.5 h at 4ºC in order to precipitate the virus particles. The solution was centrifuged at 12000 g for 20 min at 10ºC. The resulting pellet was resuspended for overnight in suspension buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) at 4ºC, which was 1/5 of the volume of the extraction buffer taken. The next day this suspension was subjected to centrifugation at 10000 g for 10 min and the supernatant was overlaid onto 6 ml of 30% sucrose solution prepared in suspension buffer and centrifuged for 3 h at 51000 g at 10ºC (Beckman Optima LE-80 K). The pellet was collected and suspended in small volume of suspension buffer. Further purification was attempted by using sucrose gradient (10-40%) where 2 ml of the virus sample was overlaid onto the preformed gradient and centrifuged at 35000 g for 3 h using SW-28 rotor (Beckman). Alternatively, the pellet obtained from 30% sucrose step was also subjected to purification by cesium chloride step gradient (0-40%) at 35000 g for 5 h using SW-41 rotor. After the run was over the gradient tubes were checked for the presence of light scattering zone containing the virus particles.

Electron microscopy

Electron microscopy of purified preparation was carried out at the Unit of Virology, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi-110 012 using negative staining with 2% uranyl acetate (pH 4.5).

Antiserum production

The antiserum against the whole virus was produced in New Zealand white rabbit. 500 μl of the virus preparation mixed with 500μl of Freund's incomplete adjuvant (1:1 v/v) was injected intramuscularly 6 times at weekly intervals. Fourteen days after the last injection, the rabbit was bled and the antiserum was collected.

Purification of Immunoglobulin G (IgG) and preparation of enzyme conjugate

The antiserum was initially cross-absorbed with pellet obtained from healthy black pepper leaves (to remove antibodies present against healthy plant proteins) before loading onto the affinity column containing Protein-A coupled to cyanogen bromide activated agarose (Genei, Bangalore) for the purification of IgG. Cross-absorbed polyclonal antiserum, 4 ml, was passed through the column and the column was washed with 25 ml of equilibration (wash) buffer to remove all the unbound materials. The IgG bound to the column was eluted by adding 5 ml of elution buffer and IgG was quantified by taking O.D. values at 280 nm (1.4 O.D. = 1mg.ml^{-1} of IgG). One milligram of this IgG was used for conjugate preparation through one-step glutaraldehyde method as described by Avrameas (1969) using the enzyme, alkaline phosphatase.

Double antibody sandwich (DAS) ELISA

DAS-ELISA was done on polystyrene (Tarsons) plate using the protocol described by Clark *et al.* (1986). Wells were initially coated with badna IgG at 1μg.ml^{-1} in coating buffer. The antigen was prepared by grinding the infected leaf tissue in 10 volumes of PBS-T containing 2% polyvinyl pyrrolidone (PVP) and 0.2% bovine serum albumin (BSA) followed by centrifugation at 8000 rpm for 30 sec. The supernatant was loaded onto the ELISA wells. The conjugate was used at 1:2000 dilutions. The ELISA plate was read at 405 nm, 90 min after the addition of the substrate (p-nitro phenyl phosphate, Genei, Bangalore) with the help of ELISA reader (μ-Quant, Biotek Instruments Inc., USA)

RESULTS AND DISCUSSION

Final purification using sucrose density gradient (10-40%) or cesium chloride step gradient (0-40 %) did not reveal the presence of light scattering zone of virus in the gradient tube. Reducing the duration of centrifugation and varying the sucrose gradient (20-60%) also did not give any light scattering zone. But the pellet in the gradient tube revealed the presence of bacilliform particles with dimensions of about 120 x 30 nm along with few host impurities indicating the presence of putative badnavirus (Fig.2). This preparation was used for the production of polyclonal antiserum in New Zealand white rabbit. The virus preparation was immunogenic and produced antiserum with a titre of 1: 8000 in ELISA. The polyclonal antiserum was cross-

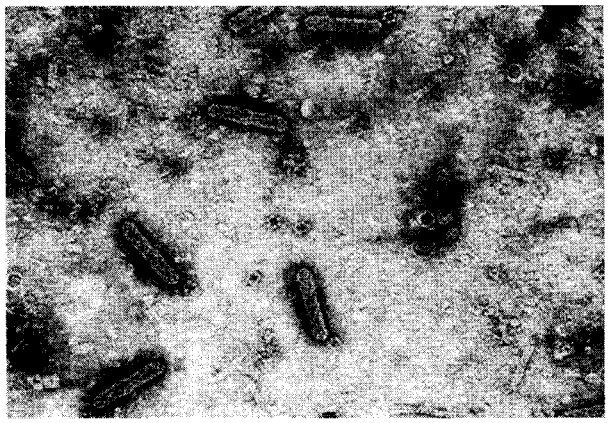

Fig.2. Electron micrograph of purified preparations showing bacilliform shaped particles. Bar represents 200 nm.

absorbed to remove antibodies against host proteins before isolating virus specific IgG. The initial experiments using various concentrations of coating antibody (IgG) and conjugate with an aim to standardize DAS-ELISA was showed 1μg.ml^{-1} of IgG coating and 1:2000 dilutions of conjugate. DAS-ELISA procedure could detect putative badnavirus infection in black pepper samples collected from different regions (Table 1). A total of seventy isolates of black pepper representing different cultivars and regions of Andhra Pradesh, Karnataka, Kerala, Tamil Nadu and West Bengal and 10 different species of *Piper* maintained in the black pepper germplasm at IISR farm, Peruvannamuzhi, were used in the tests. Among these, 17 isolates and four different species of *Piper* showed positive reaction in DAS-ELISA (Table 1). The variations in the O.D. values obtained with different samples indicate the varying concentration of the virus. However the severity of the disease symptoms could not be correlated with the OD values obtained. The Badnavirus, PYMV is known to infect *P. nigrum* and *P. betle* in Malaysia, Philippines, Sri Lanka and Thailand (Lockhart *et al.*, 1997). The detection of putative badnavirus on *P. colubrinum*, *P. hapnium, P. longum* and *P. mullesua* in the present investigations is the first report from India and elsewhere. Among these, *P. longum* is cultivated for medicinal use while *P. colubrinum* is used as rootstock to get *Phytophthora* resistant black pepper while other species may be of use in breeding to improve black pepper. However the exact taxonomic identity of badnavirus infecting black pepper and other *Piper* spp. in India is yet to be determined.

As black pepper is vegetatively propagated, it is essential to develop methods for producing and certifying virus-free planting material. DAS-ELISA was reported to be sensitive and suitable for specific detection of many plant viruses (Hull, 2002). DAS –ELISA was successful in the detection of CMV infecting black pepper (Bhat *et al.*, 2004). In the present study, though the healthy black pepper samples gave a faint colour indicating background reaction, the infected samples could be distinguished from the healthy samples with an increase in the O.D. values. However, some of the samples exhibiting symptoms of the disease gave an O.D. close to that of the healthy samples (Table 1). This could be due to low titre of the virus in these samples. This kind of close values to that of healthy were also reported in the detection of PYMV infecting black pepper in Sri Lanka (de Silva *et al.*, 2002). Other members of the badnavirus group such as *Sugarcane bacilliform virus* (SCBV), *Rice tungro bacilliform virus* (RTBV) and *Cocoa swollen shoot virus* (CSSV) also tend to have low absorbance values. Hence more sensitive and specific detection technique like PCR may be necessary for samples that were negative in DAS-ELISA. However due to its cost effectiveness and adaptability in testing large number of samples, ELISA can be used for primary screening of samples. Our initial efforts to detect the putative badnavirus infecting black pepper through PCR using published badnavirus specific degenerate primers (known to amplify wide range of badnaviruses) such as BADNA T + BADNA 2, BADNA T + BADNA 3, BADNA 2 + MYS 3 (Lockhart *et al.*, 1997) and BADNA T + SCBV R_1 (de Silva *et al.*, 2002) was unsuccessful due to the presence of bands both in healthy and infected black pepper extracts. Efforts are currently on to clone, sequence and design primers for the specific detection of putative badnavirus infecting black pepper.

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