Partial characterization and identification of a virus associated with stunt disease of black pepper (*Piper nigrum*) in South India

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A virus associated with stunt disease of black pepper (*Piper nigrum*) in South India was purified and partially characterized. The virus was transmitted through stem cuttings of black pepper and by grafting. It was also sap-transmitted with difficulty from black pepper to black pepper and to a few experimental plants. *Chenopodium amaranticolor*, *C. quinoa*, *Vigna unguiculata*, *V. radiata* and *V. mungo* reacted with local chlorotic/necrotic lesions. *Cucumis sativus* and five tested solanaceous plants reacted with systemic mosaic symptoms. The leaf extract of diseased black pepper or virus purified from diseased black pepper leaves in direct antigen coating-ELISA and electroblot immunoassay tests reacted positively with polyclonal antisera of cucumber mosaic cucumovirus (CMV)-Banana (India), CMV-Brinjal (India), CMV-Chilli (India), CMV-Tomato (India), CMV-L (USA) and CMV-A (China). The negatively stained purified virus preparation contained non-enveloped isometric virions. The M_r of double-stranded (ds) RNAs isolated from infected black pepper leaves was 2.42, 2.20 and 1.62×10^6 Da. The coat protein of disrupted purified virions in 12% SDS-PAGE was resolved into one major polypeptide with M_r 26.1 kDa and of nucleic acid in 1% agarose gel resolved into four species with M_r 1.21, 1.10, 0.81 and 0.37×10^6 Da. Particle morphology, antigenic relationships with CMV, coat protein and genome characteristics suggest that the virus associated with stunt disease of black pepper in the state of Kerala, South India could be an isolate of CMV.

IN India, black pepper (*Piper nigrum*) is grown over an area of 1,95,050 ha, traditionally in Kerala, Karnataka and Tamil Nadu, but has been recently introduced in other states like Andhra Pradesh and Orissa during 1995–1996 (ref. 1). The stunt disease occupied the third place among serious diseases of black pepper[2]. Diseases of a similar nature (sickle leaf, wrinkled leaf, dog's ear) were also reported from Malaysia, Indonesia, Sri Lanka, Thailand and Brazil[3–8]. At the 1991 International Workshop on Black Pepper Diseases held at Lampung, Indonesia, scientists designated the diseases as 'Stunted Disease of Black Pepper' for uniform terminology[9].

In India, stunt disease was first reported in a Government Black Pepper Nursery at Neriamangalam in Idukky district of Kerala during 1975 (ref. 10). Subsequently, a black pepper mosaic was reported from Tamil Nadu[11]. Since then stunt disease has been recorded from almost all pepper-growing regions in South India. It is thought that its vertical transmission through stem cuttings could be the reason for its widespread occurrence.

The etiology of the disease was uncertain, but suspected to be caused either by a MLO or a virus. In Brazil, it was suggested to adopt plant quarantine regulations to avoid the dissemination of cucumber mosaic cucumovirus (CMV), within the country and to other South American countries, through stem cuttings of black pepper[6]. Attempts were made to identify the virus(es) causing stunt disease of black pepper in Sarwak, Malaysia[12]. It was reported that the symptoms were caused by a complex of viruses (a badnavirus, a spherical virus and a clostero-like virus particle). Further, it was suggested that CMV is not the causal agent of the disease, as virus particles were not observed by immunospecific electron microscopy using antisera of CMV S96 2C, CMV-B and CMV-C. Subsequently, Lockhart *et al.*[8] reported a new mealybug-transmitted *Piper* yellow mottle badnavirus from diseased black pepper exhibiting chlorotic mottling, chlorosis, leaf distortion and reduced plant vigour and fruit set in Malaysia, Thailand, The Philippines and Sri Lanka. In this paper we report the partial characterization of a virus associated with stunt disease of black pepper in South India and its identification as an isolate of CMV.

Scions from diseased black pepper plants (cv. Imperian) collected from the Pulpally area of Wyanad district, Kerala during 1995, were either side-cleft or top-cleft grafted to healthy pepper plants as described by Bos[13]. Subsequently, the virus was maintained in black pepper by vegetative propagation. For sap inoculation, young symptomatic leaves collected from diseased black pepper plants were macerated in a cold mortar using cold 0.1 M potassium phosphate buffer, pH 7.0, containing 0.1% 2-mercaptoethanol (1 g leaf material/10 ml buffer). The carborundum (600 mesh) abrasive dusted young leaves of healthy black pepper plants were pin-pricked and immediately rubbed with muslin cloth pad soaked in the freshly prepared inoculum.

Eighteen species belonging to four dicotyledonous families were mechanically inoculated with sap extracted from black pepper leaves as described above and later developing leaves were tested for the presence of virus by direct antigen coating–enzyme linked immunosorbent assay (DAC–ELISA)[14], using heterologous antiserum (CMV-Banana) and by back-inoculation to local lesion assay plant (*Vigna radiata*).

The virus from fresh young symptomatic leaves of infected black pepper plants was purified essentially as

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per the procedure of Walkey[15]. The infectivity of purified virus was checked by rubbing it on the pin-pricked leaves of healthy pepper plants as described above. The virus quality and quantity were measured based on its UV-light absorption characteristics determined by Hitachi UV-visible spectrophotometer. The virus concentration was determined by considering A_{260} 5.0 = 1 mg/ml[16]. The purified virus preparation was stained with 1% aqueous uranyl acetate, viewed in a Philips 201 C transmission electron microscope and photographs were taken.

DAC–ELISA and electroblot immunoassay (EBIA)[14,17] were employed to determine the antigenic relationships of the virus with polyclonal antisera of CMV-L (Demski, J. W., University of Georgia, USA), CMV-A (Barnett, O. W., Clemson University, USA), CMV-Banana, CMV-Chilli, CMV-Tomato and CMV-Brinjal isolates from Andhra Pradesh (Sreenivasulu, P., S.V. University, India). In DAC–ELISA the plates were coated with leaf antigen samples extracted in carbonate buffer (1 g/9 ml, 10^{-1} dilution). Crude antisera at 1 : 500 dilution and alkaline phosphatase-labelled goat antirabbit antibodies (Genei, Bangalore) at 1 : 1000 dilution in PBS-TPO were used in the subsequent steps. Finally *p*-nitrophenyl phosphate (Sigma) substrate at 5 mg/10 ml was added. The plate was then incubated in dark at room temperature for 1 h. The reactions were terminated by adding 50 µl 3 N NaOH to each well and the absorbance recorded by reading at A_{405} in Bio-Tek Ceres 900 ELISA Plate Reader. For EBIA, the coat protein of the purified virions was separated by SDS-PAGE[18] and the resolved proteins electroblotted (200 mA, 3 h) onto nitrocellulose membrane (Bio-Rad) using wet-blot apparatus (Broviga, Chennai). The transferred proteins were probed with antisera (1 : 500 dilution) and antigen–antibody reactions were detected using horseradish peroxidase-labelled goat antirabbit FC-specific antibodies as secondary antibody at 1 : 1000 dilution and 3,3′-diaminobenzidine (DAB) as substrate (Genei, Bangalore).

The M_r of the virus coat protein of disrupted purified virions was determined by 12% SDS-PAGE[18]. The marker proteins (Sigma) used are albumin bovine (66 kDa), albumin egg (45 kDa), carbonic anhydrase (29 kDa), β-lactalbumin (18.4 kDa) and lysozyme (14.3 kDa). Electrophoresis was carried out in a mini slab gel apparatus at 100 V for 2 h. The M_r of the virus protein was calculated from standard curve prepared by plotting distance migration of marker proteins against M_r, on a semi-log graph paper.

The purified virions were disrupted in the disruption buffer of Woods and Coutts[19] and the released nucleic acid was analysed by horizontal agarose (10 g/l) gel electrophoresis[20]. Electrophoresis was carried at 60 V for 2 h and the ethidium bromide-stained gel was viewed on a UV-transilluminator. TMV-RNA (2.0×10^6 D) and *E. coli* ribosomal RNA (0.56 and 1.1×10^6 D) were used as markers. The dsRNA was isolated from infected pepper leaves by lithium chloride fractionation method of Diaz-Ruiz and Kaper[21] and analysed by agarose (20 g/l) gel electrophoresis using TBE buffer at 60 V for 2 h (ref. 20). The gel stained with ethidium bromide was viewed on a UV-transilluminator. Lambda phage DNA/*Hin*dIII digest (Genei, Bangalore) was used as marker. The M_r of viral RNA and dsRNA was calculated from a standard curve prepared by plotting distance migration against M_r on a semi-log graph paper.

The observed interveinal yellow flecking, yellow mottling, dark-green vein banding, narrow distorted leathery leaves and overall reduction in the growth of the infected black pepper in the Wyanad district of Kerala, are similar to symptoms described on black pepper in several south-east Asian countries[3,8,12]. Previous reports from south-east Asian countries indicate that stunt disease of black pepper with varied foliar symptoms is a disease complex caused by more than one type of virus[8,12]. Even though the stunt disease is the third most important disease of black pepper in South India, efforts to characterize and identify the causal agent(s) have not been successful until now.

A sap transmissible virus associated with stunt diseased black pepper in Kerala was transmitted to a few experimental hosts with difficulty. *Chenopodium amaranticolor*, *C. quinoa*, *Vigna unguiculata*. *V. mungo* and *V. radiata* reacted with local chlorotic/necrotic lesions and *Cucumis sativus*, *Capsicum annuum*, *Nicotiana tabacum* var. Harrison special, *N. rustica*, *Petunia hybrida* and *Physalis minima* reacted with systemic mosaic symptoms (Table 1).

Table 1. Reaction of different host plants to sap inoculation with the extract of *Piper nigrum* leaves

Host plant	Type of reaction
Chenopodiaceae	
Chenopodium amaranticolor	LCL
C. quinoa	LCL
Cucurbitaceae	
Cucumis sativus	SM
Cucurbita pepo	NI
Luffa acutangula	NI
Momordica charantia	NI
Fabaceae	
Arachis hypogaea	NI
Cajanus cajan	NI
Dolichos lablab	NI
Glycine max	NI
Phaseolus vulgaris	NI
Vigna mungo	LNL
V. radiata	LNL
V. unguiculata	LNL
Solanaceae	
Capsicum annuum	SM
Nicotiana rustica	SM
N. tabacum var. Harrison special	SM
Petunia hybrida	SM, LD
Physalis minima	SM

LCL, Local chlorotic lesions; LNL, Local necrotic lesions; LD, Leaf distortion; NI, Not infected; SM, Systemic mosaic.

The virus did not infect *Cucurbita pepo*, *Luffa acutangula*, *Momordica charantia*, *Arachis hypogaea*, *Glycine max*, *Dolichos lablab* and *Phaseolus vulgaris*. No virus was detected in any of these hosts, either by DAC–ELISA or bioassay to the local lesion host, despite using an antioxidant (2-mercaptoethanol) in the extraction buffer. The success of mechanical transmission of the virus by rubbing the fresh extracts of diseased black pepper leaves is low as the leaf extracts contained phenolic compounds. Similar problems were also encountered in transmitting the viruses mechanically from black pepper to other hosts[8,12].

The purified virus was banded as faint single light-scattering zone in the gradients. Its $A_{260/280}$ and $A_{max/min}$ ratios (1.72 and 1.25, respectively) are similar to values reported for CMV[22]. The uranyl acetate-stained purified virus preparations contained a few non-enveloped isometric particles. Healthy black pepper, mechanically inoculated with purified virus preparation showed systemic yellow mosaic symptoms.

Table 2. Reaction of a virus associated with stunt disease of *Piper nigrum* with different antisera in DAC–ELISA

	Piper nigrum	
Antiserum	Healthy	Infected
CVM-L (USA)	0.12	1.65*
CMV-A (China)	0.08	3.87
CMV-Banana (India)	0.19	3.41
CMV-Brinjal (India)	0.05	3.35
CMV-Tomato (India)	0.02	1.93
CMV-Chilli (India)	0.07	2.20

*A_{405} values represent average of three wells taken one hour after adding the substrate.

The black pepper virus isolate in DAC–ELISA and EBIA tests positively reacted with polyclonal antisera of six CMV strains/isolates (Table 2; Figure 1), indicating that the virus is antigenically related to CMV. It also reacted in DAC–ELISA (Penicillinase system) with peanut stunt virus (PSV) (results not shown) which is serologically related to CMV[25]. Further, our initial attempts to characterize and identify the virus(es) in the stunt diseased black pepper leaf extract failed to react in DAC–ELISA with antisera of two potyviruses (tobacco etch and datura leaf distortion viruses) and tobacco mosaic virus (tobamovirus), indicating that flexuous filamentous and rigid rod-shaped particles reported on *P. betle* in India are probably absent in *P. nigrum*[23,24].

The coat protein of disrupted virions in SDS-PAGE was resolved into one species with M_r of 26.1 kDa (Figure 2), which is close to values reported for several isolates of CMV[25]. The genomic nucleic acid of the black pepper virus isolate in agarose gel was resolved into four species with M_r of 1.21, 1.10, 0.81 and 0.37×10^6 Da, respectively. The fourth species probably represents a subgenomic RNA[25].

Analysis of suspected virus-diseased leaf samples for dsRNA helps to predict the type of virus involved and is a useful initial approach in virus identification studies[26]. Pares *et al.*[27] have differentiated several biologically dis-

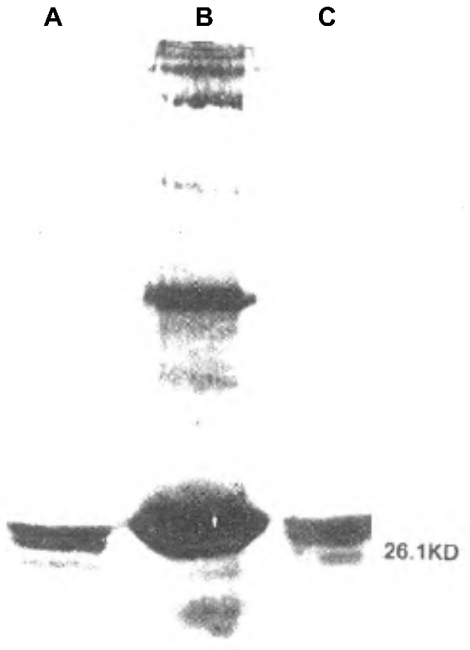

Figure 1. EBIA analysis of coat protein of a virus associated with diseased black pepper. Blotted virus protein probed with antisera of CMV-A (lane A), CMV-Banana (lane B) and CMV-L (lane C).

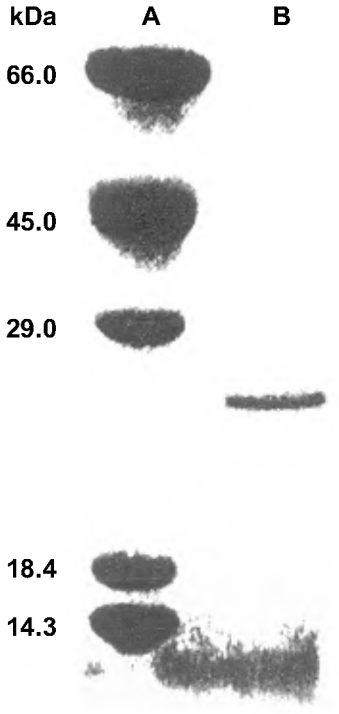

Figure 2. SDS-PAGE analysis of coat protein of a virus purified from diseased black pepper. Lane A, Marker proteins; lane B, Virus protein.

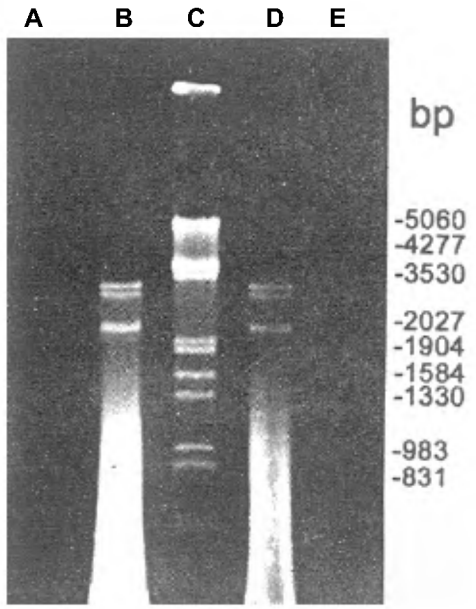

Figure 3. Agarose gel electrophoretic analysis of dsRNA isolated from black pepper leaves. Lanes A and E, dsRNA isolated from healthy leaf tissue; Lanes B and D, dsRNA isolated from infected leaf tissue; Lane C, Marker DNA.

tinct CMV isolates occurring in Australia by PAGE analysis of dsRNA. The dsRNA isolated from diseased black pepper leaves in agarose (20 g/l) gel was resolved into three species with M_r of 2.42, 2.20 and 1.62 × 10^6 Da (Figure 3). These values are twice the M_r of the corresponding single-stranded genomic RNAs. The number of dsRNA and ssRNA species and the M_r of the black pepper virus isolate are similar to the values reported for CMV isolates[25]. Lockhart *et al.*[8] have reported the association of three dsRNA species with 30 nm diameter particles found in black pepper samples from Sri Lanka, but not directly in the diseased leaf samples.

The information presented on limited host-range, particle morphology, antigenic relationships, coat protein, dsRNA and genomic nucleic acid suggests that the virus associated with black pepper in Kerala may be an isolate of CMV (CMV-black pepper). This agrees with other workers who have suggested to follow quarantine regulations to avoid the dissemination of CMV through stem cuttings of black pepper[6]. However, the viruses reported from south-east Asian countries[8,12] in the diseased black pepper in South India need to be investigated to understand their role, if any, in the induction of the stunt disease.

1. Anon, *Spices Statistics*, Spices Board, Ministry of Commerce, Government of India, Cochin, India, 1996.
2. Sarma, Y. R., Ramachandran, N. and Anandraj, M., in Disease of Black Pepper – Proceedings of the International Pepper Community Workshop on Joint Research for the Control of Black Pepper Diseases (eds Sarma, Y. R. and Premkumar, T.), National Research Centre for Spices, Calicut, India, 1991, pp. 55–101.
3. Holliday, P., *Common. Phytopathol. News*, 1959, **5**, 49–54.
4. Sitepu, D. and Kasim, R., in Diseases of Black Pepper – Proceedings of the International Pepper Community Joint Research for the Control of Workshop on Black Pepper Diseases (eds Sarma, Y. R. and Premkumar, T.), National Research Centre for Spices, Calicut, 1991, pp. 13–28.
5. Randombage, S. and Bandara, J. M. R. S., *Plant Pathol.*, 1984, **33**, 479–482.
6. Duarte, M. L. R. and Albuquerque, F. C., in Disease of Black Pepper – Proceedings of the International Pepper Community Workshop on Joint Research for the Control of Black Pepper Diseases (eds Sarma, Y. R. and Premkumar. T.), National Research Centre for Spices, Calicut, India, 1991, pp. 39–54.
7. Ben, F. A., Susango, R. and Sitepu, D., in Proceedings of the International Workshop on Black Pepper Diseases (eds Sitepu, P. and Ujang Superman, D.), Agency Research and Development Research Institute for Spices and Medical Crops, Bogor, Indonesia, 1992, pp. 220–226.
8. Lockhart, B. E. L., Kiltisak, K. A., Jones, P., Padmini, D. S., Olsziewski, N. E., Lockhart, N., Nuarnchan, D. and Sangalang, J., *Eur. J. Plant Pathol.*, 1997, **103**, 303–311.
9. Wahid, P., Sitepu, D., Deciyanto, S. and Ujang Superman, D., in Proceedings of the International Workshop on Black Pepper Diseases (eds Sitepu, P. and Ujang Superman, D.), Agency for Agricultural Research and Development Research Institute for Spices and Medical Crops, Bogor, Indonesia, 1992.
10. Paily, P. V., Remadevi, L., Nair, V. G., Menon, M. R. and Nair, M. R. G. K., *J. Plant. Crops*, 1981, **9**, 61–62.
11. Prakasam, V., Subbraja, K. T. and Bhaktavastalu, C. M., *Indian Cocoa, Arecanut Spices J.*, 1990, **13**, 104.
12. Eng, L., Jones, P., Lockhart, B. and Martin, R. R., in *Pepper Industry: Problems and Prospects* (eds Ibrahim, M. Y., Rang, C. F. J. and Ipor, I. B.), University Pertanian, Malaysia Bintulu Campus, Bintulu, Malaysia, 1993, pp. 149–161.
13. Bos, L., in *Methods in Virology* (eds Marmorosch, K. and Koprowski, H.), Academic Press, New York, 1967, vol. 1, pp. 403–410.
14. Hobbs, H. A, Reddy, D. V. R, Rajeswari, R. and Reddy, A. S., *Plant Dis.*, 1987, **71**, 744–749.
15. Walkey, D. G. A., *Applied Plant Virology,* Wiley and Sons, New York, 1985, p. 329.
16. Brunt, A. and Crabtree, K., *Viruses of Tropical Plants*, CAB International, Wellingford, UK, 1990, p. 707.
17. Burgermeister, W. and Koenig, R., *Phytopathol. Z.*, 1984, **111**, 15–25.
18. Laemmli, U. K., *Nature*, 1970, **227**, 680–685.
19. Woods, K. R. and Coutts, R. H. A., *Physiol. Plant Pathol.*, 1975, **1**, 139–145.
20. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning – A Laboratory Manual*, Cold Spring Harbor Press, New York, 1989, 3 volumes.
21. Diaz-Ruiz, J. R. and Kaper, T. M., *Prep. Biochem.*, 1978, **8**, 1–17.
22. Lot, H., *Arch. Virol.*, 1991, **2**, 386–388.
23. Singh, S. J. and Raghavendra Rao, N. N., *Curr. Sci.*, 1988, **57**, 1024–1025.
24. Johri, J., Srivastava, K. M., Raizada, R. K., Deshpande, A. L. and Singh, B. P., *Indian Phytopathol.*, 1990, **43**, 491–495.
25. Palukaitis, P., Rossinek, M. J., Deitzen, R. G. and Francki, R. I. B., *Adv.Virus Res.*, 1992, **41**, 281–348.
26. Dodds, J. A., in *Diagnosis of Plant Virus Diseases* (ed. Matthews, R. E. F.), CRC Press, Boca Raton, 1993, pp. 273–294.
27. Pares, R. D., Gillings, M. R. and Gunn, I. V., *Intervirology*, 1992, **34**, 23–29.

Received 18 August 2000; revised accepted 20 October 2000