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Association of a badnavirus in black pepper (*Piper nigrum* L.) transmitted by mealybug (*Ferrisia virgata*) in India

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The association of a badnavirus with disease-affected black pepper leaf samples collected from Kozhikode (Calicut) and Wyanad districts of Kerala was established on the basis of symptomatology, vector transmission, electron microscopy and serology. The virus induces vein clearing, chlorotic flecks, chlorotic mottling along veins and characteristic curling of leaves leading to reduced vigour and yield. The virus was transmitted from diseased to healthy black pepper plants by grafting and mealybug (*Ferrisia virgata*). The virus could also be transmitted by mechanical means with difficulty to black pepper, but not to other hosts tested. The virus showed positive serological relationship with Banana streak virus (BSV) and Sugarcane bacilliform virus (ScBV) in direct antigen-coated enzyme-linked immunoassay (DAC-ELISA) using polyclonal antisera. The exact taxonomic identity of the virus remains to be determined.

BLACK pepper, obtained from dried berries of *Piper nigrum* L., is an important condiment of international commerce for India, earning around Rs 88 crores annually through export. India is a leading producer of black pepper in the world and the crop is grown in an area of 1.92 lakh hectares, with a production of 30.23 lakh tons annually[1]. The crop is mainly grown in Kerala and Karnataka. However, the productivity of the crop is considerably low due to many biotic stresses, including viruses. Viruses belonging to genera *Badna*, *Cucumo* and *Clostero* have been recorded on black pepper[2–6]. The disease caused by Cucumber mosaic virus (CMV) (genus: *Cucumo*) 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[2,5,6]. The disease caused by Piper yellow mottle virus (PYMV) (genus: *Badna*) is characterized by chlorotic mottling, chlorosis, vein clearing, leaf distortion, reduced plant vigour and poor fruit set[2,4]. PYMV has been reported from Brazil, Malaysia, Thailand, Philippines and Sri Lanka[2,4].

In India, only the association of a CMV has been established with stunted disease-affected black pepper samples[5]. In addition, a mosaic disease on black pepper was observed in serious proportions in parts of Kerala for the past few years. Up to 100% incidence of this disease has been reported in certain black pepper plantations, especially in Kozhikode and Wyanad districts. The disease is characterized by vein clearing, scattered chlorotic flecks (Figure 1 *a*) followed by chlorotic mottling along veins leading to interveinal chlorosis and characteristic curling of the leaves (Figure 1 *b*). In a few cultivars, vein banding, vein thickening and green island-like symptoms are also seen. The infected vines had reduced vigour and yield. Though the disease has been noticed on all the cultivars, its incidence and severity was more on Karimunda. Since it has not been established so far, we report the results of our studies which revealed the association of a badnavirus based on its transmission, electron microscopy and serological characteristics.

The virus isolate was collected from black pepper vines from plantations in Kozhikode and Wyanad districts, including the experimental farm at the Indian Institute of Spices Research (IISR), Peruvannamuzhi during April–May 2002. The isolate was maintained on black pepper by vegetative propagation under insect-proof glasshouse conditions at 25–28°C. For mechanical inoculation, the inoculum was prepared by extracting the sap using chilled 0.1 M phosphate buffer (pH 7.2) containing 0.1% 2-mercaptoethanol poured in mortar kept in an ice tray. The inoculum was rubbed on the leaves of test plants dusted with celite or carborundum powder. For host-range studies, plants belonging to four families namely, Cucurbitaceae (*Cucumis sativus*, *C. pepo*), Fabaceae (*Cajanus cajan*, *Glycine max*, *Vigna mungo*, *V. radiata*, *V. unguiculata*), Poaceae (*Zea mays*) and Solanaceae (*Nicotiana benthamiana*, *N. glutinosa*, *N. tabacum*, *Physalis floridana*) were grown in pots raised under insect-proof glasshouse, and were rub-inoculated. Ten plants of each species were inoculated and kept under observation for two months. For graft transmission, scions from dis-

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eased black pepper plants were top-cleft grafted to healthy black pepper plants raised from seeds, as described earlier[7].

Mealybug transmission tests were done using *Ferrisia virgata* (Cockerell) (Family: Pseudococcidae) commonly seen on shoots of black pepper plants (Figure 2). The adults were collected from black pepper plants (at IISR, Kozhikode) and reared on matured pumpkins in the laboratory. After three generations on the pumpkin, the non-viruliferous, young adult female mealybugs were given a 24 h acquisition access on symptomatic black pepper leaves (on the lower surface) kept in a petri plate lined with moist filter paper and covered with black cloth. Ten mealybugs each were then transferred to 20 healthy test seedlings of black pepper cv. Karimunda at the four-leaf stage, kept in a cage covered with black cloth. After an inoculation access period of 24 h, the plants were sprayed with insecticide (chloropyriphos @ 0.075%). The seedlings were then removed from the cage and kept for observation in the insect-proof glasshouse.

Direct antigen-coated enzyme-linked immunoassay (DAC-ELISA)[8] was performed using antisera to badna-, cucumo-, ilar-, poty- and tospo-viruses. Polyclonal antisera to different badna viruses, namely Commelina yellow mottle virus (CoYMV), Banana streak virus (BSV), Rice tungro bacilliform virus (RTBV) and Sugarcane bacilliform virus (ScBV) and, antiserum to a potyvirus (Potato virus *Y*, PVY), ilarvirus (Tobacco streak virus, TSV)

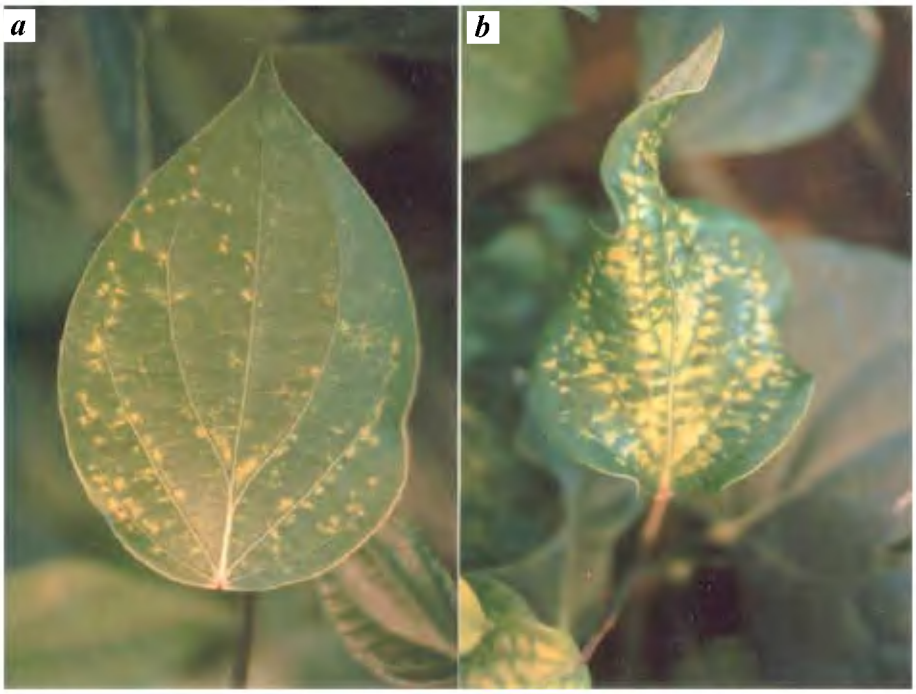

Figure 1. Disease-affected black pepper leaf showing vein clearing and chlorotic flecks (***a***), and chlorotic mottling along veins and characteristic curling of leaves (***b***).

Figure 2. Mealybug, *Ferrisia virgata* used in the transmission tests.

Figure 3. Mealybug-transmitted black pepper seedling exhibiting initial symptoms of the disease.

and tospovirus (Groundnut bud necrosis virus, GBNV) were obtained from Virology Unit, Indian Agricultural Research Institute (IARI), New Delhi. Antiserum to CMV was obtained from Bio-Rad, Phytodiagnostics, France. The assay was performed on polystyrene plate (Co-Star). Infected and healthy leaf tissues were triturated at 1 : 5 dilution in coating buffer containing 2% polyvinyl pyrrolidone (PVP, MW 40,000). These extracts were centrifuged briefly and the supernatant was placed into three wells per sample and incubated at 37°C for 1 h. Polyclonal rabbit antisera were used at 1 : 1000, except for CMV which was used at 1 : 100 (as per supplier's instructions), and antirabbit immunoglobulin-alkaline phosphatase conjugate (Sigma, St. Louis, USA) was used at 1 : 20,000. Absorption values at 405 nm were recorded using a microplate reader (Bio-Tek Instruments), 1 h after adding the substrate, *p*-nitro phenyl phosphate (0.5 mg/ml of substrate buffer). Electron microscopy of infected leaves was carried out at the Virology Unit, IARI and at the Central Plantation Crops Research Institute, Regional Station, Kayamkulam, using negative staining with 2% uranyl acetate (pH 4.5).

The graft-transmitted black pepper plants showed typical symptoms of the disease in 2–3 months. The disease could also be transmitted with difficulty by mechanical inoculation onto black pepper and only 1 of 10 healthy seedlings showed typical symptoms of the disease. No local or systemic symptoms appeared in any of the other hosts tested.

The disease could easily be transmitted by the mealybug, *F. virgata*, from naturally diseased black pepper to healthy seedlings of black pepper cv. Karimunda. The initial symptoms of the disease, like vein clearing and chlorotic mottle could be seen in 14 of 20 test plants in 5 weeks after inoculation (Figure 3). The symptoms were similar to those observed under natural conditions. The presence of virus in these plants was also confirmed through ELISA tests.

In DAC-ELISA, none of the samples reacted with CMV, GBNV, PVY and TSV antisera, suggesting the lack of association of a cucumo-, tospo-, poty- or ilarviruses with the disease. Further, among the antisera to different badnaviruses tested, none of the samples reacted with RTBV and CoYMV antisera, while all the samples reacted with BSV and ScBV antisera suggesting the association of a badnavirus serologically related to BSV or ScBV (Table 1). PYMV antiserum could not be used in the tests because of its non-availability. Majority of the samples reacted more strongly with BSV antiserum, suggesting close antigenic relationship between black pepper badnavirus and BSV (Table 1). These data were also supported by the electron microscopy of leaf dip preparations of diseased leaves, which showed the presence of bacilliform-shaped particles measuring about 30 nm × 120 nm in size, although particle concentration was very low (one particle per 4–5 squares of the grid; Figure 4).

Table 1. Detection of badnavirus in field-infected black pepper plants in direct antigen-coated (DAC) ELISA*

	A_{405} value against antisera to	
Place of collection	BSV	ScBV
Kozhikode		
Sample 1	0.17	0.10
Sample 2	0.19	0.11
Sample 3	0.33	0.09
Sample 4	0.56	0.12
Sample 5	0.11	0.11
Sample 6	0.22	0.19
Sample 7	0.19	0.11
Sample 8	0.20	0.13
Wyanad		
Sample 1	0.10	0.13
Sample 2	0.48	0.19
Sample 3	0.44	0.28
Sample 4	0.12	0.16
Sample 5	0.10	0.09
Sample 6	0.22	0.19
Sample 7	0.26	0.21
Sample 8	0.31	0.19
Healthy black pepper	0.04	0.07

*Average of three replications, 1 h after substrate addition.
BSV, *Banana streak virus*; ScBV, *Sugarcane bacilliform virus*.

Figure 4. Electron microscopy of leaf dip preparation of diseased black pepper leaf showing a bacilliform-shaped particle.

The disease symptoms, such as mild to severe chlorotic flecking, vein clearing, interveinal chlorosis, leaf deformation and reduction in vigour and yield observed on

black pepper vines in Kozhikode and Wyanad districts of Kerala are similar to the symptoms described on black pepper in several South East Asian countries[4] and Brazil[2]. In both these areas, the causal virus has been identified as a badna virus PYMV[4]. PYMV had non-enveloped bacilliform virions and was transmitted by mechanical inoculation and by citrus mealybug, *Planococcus citri*[4]. However, in Brazil it is suspected to be transmitted by another species of mealybug, *Pseudococcus elisae*[2]. Further, PYMV was also serologically closely related to both BSV and ScBV[4]. Our studies based on symptomatology, serological affinities, electron microscopy and mealybug transmissibility, clearly indicate that the virus under investigation is a member of badnavirus. Although the present virus could be transmitted by *F. virgata*, a common foliar mealybug found associated with black pepper in Kerala and Karnataka, it remains to be seen whether this virus could also be transmitted by other species, as nine species of mealybugs have been reported to be associated with black pepper in India[9]. This is a report of the transmission of a badnavirus by *F. virgata* in black pepper. However, it is known to transmit a badnavirus in cacao (Cacao swollen shoot virus, CSSV) in Africa[10].

Badnaviruses have been reported from banana[11], citrus[12], rice[13] and sugarcane[14] in India. In most cases, black pepper is grown as a mixed crop with other badnavirus-susceptible crops like banana and cacao. Hence it is important to study whether these crops are a source of infection to black pepper or vice versa. To unequivocally identify whether badnavirus on black pepper is a strain of an already known badnavirus or a distinct badnavirus, the virus will have to be further characterized at biological and molecular levels.

Occurrence of CMV on black pepper in India is already known[5], and the occurrence of mixed infections by both these viruses cannot be ruled out. As black pepper is clonally propagated, disease spread is rapid through planting material when infected plants are used as a source of planting material. As symptoms alone cannot be used as a criterion for confirming the disease-free nature of the material, it is necessary to develop methods for quick, reliable and sensitive detection (either serological or nucleic-acid based) of the virus for producing and certifying virus-free planting material.

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Oligophilic bacterial diversity of Leh soils and its characterization employing ARDRA

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Oligophiles from uncultivated, mustard-, potato- and wheat-cultivated soils of Leh were studied. The substrate concentration of the standard plate count (SPC) medium was 1.75–0.0035% to facilitate the growth of copiotrophs and oligophiles. The ratio of bacterial counts on 1/20th dilution to full strength SPC was 3.26 in uncultivated and 1.74, 1.83 and 2.7, in wheat-, mustard- and potato-cultivated soil, respectively. ARDRA pattern with *Hae*III of 36 randomly picked isolates showed a dissimilarity coefficient of 3.0. Despite the nature of the soil, seven phylogenetic groups were formed, which consisted of isolates from higher dilutions (1/20 and 1/100). Only three isolates recovered on normal SPC showed similarity with these isolates. This confirms that bacteria growing at higher dilu-

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