Research Article

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Further evidence of true seed transmission of *Piper yellow mottle virus* in black pepper (*Piper nigrum* L.)

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Abstract

A study was conducted to detect the presence of *Piper yellow mottle virus* (PYMoV) in pollen and different parts of black pepper berries. Total DNA isolated from anthers, embryo, endosperm and perisperm of berries from PYMoV infected and healthy black pepper plants were subjected to polymerase chain reaction (PCR) using PYMoV specific primers. PCR results clearly indicated the presence of PYMoV in embryo, endosperm and perisperm of berries from infected plants of all the three varieties tested (Panniyur-1, IISR-Thevam, and Subhakara) and in anthers of var. Panniyur-1 but was absent in all these parts of healthy plants. The identity of the PCR product was confirmed by sequencing. Seedlings raised from infected berries were symptomatic and were PCR positive. The present study confirmed true seed transmission of PYMoV and the possibility for pollen transmission of the virus.

Keywords: Black pepper, PCR, *Piper yellow mottle virus*, pollen transmission, seed transmission

Introduction

Black pepper (*Piper nigrum* L.), 'the king of spices' is an important commercial crop and a well-known spice in the culinary circle of India as well as abroad. Western Ghats of Kerala state is the center of origin for the crop and it spread from India to Indonesia and other countries (Ravindran, 2000). Black pepper of commerce is the mature dried berry of the pepper plant, which has got a characteristic pungency, aroma and flavours. The cultivation of the plant is threatened by many diseases, which lead to extensive crop loss. Infections caused by viruses are difficult to control and are transferred by vegetative propagation. Viruses of black pepper have been identified and characterized, which include *Piper yellow mottle virus* (PYMoV) and *Cucumber mosaic virus* (CMV) (Sarma *et al.*, 2001; Bhat *et al.,* 2003; Hareesh and Bhat 2008).

PYMoV is a member of the genus *Badnavirus*, known to infect black pepper in India and other black pepper growing South East Asian countries and Brazil (Lockhart *et al.,* 1997). The virus induces chlorotic mottling, vein clearing, leaf distortion, reduced plant vigor and poor fruit set in affected black pepper plants (Bhat *et al.,* 2003). PYMoV is known to be transmitted primarily through vegetative means (stem cuttings) while secondary spread occur in field through various species of mealybugs. Though de Silva *et al.* (2002) observed lack of seed transmission of PYMoV in black pepper, recent studies from India reported the occurrence of seed transmission of PYMoV (Hareesh and Bhat, 2010). However, information on location of PYMoV in different parts of berry and pollen is lacking. In view of this a study was conducted to determine the presence or absence of PYMoV in different parts of the seed and in anthers of black pepper.

Materials and methods

Identification of source plants

Black pepper berries were collected from healthy and infected plants of the varieties such as Panniyur-1, IISR-Thevam and Subhakara from the

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Experimental Farm of Indian Institute of Spices Research, Peruvannamuzhi, Kozhikkode. Infected and healthy plants were identified by PCR using PYMoV specific primers. Anthers of black pepper flowers were collected from PYMoV infected Panniyur-1 plants from three different areas of Kodagu, Karnataka. Leaf samples were collected from five black pepper plants of each variety and total DNA isolated were subjected to PCR using PYMoV specific primers AIB 35/AIB 36 to identify infected and healthy plants for collection of berries (Table 1).

Separation of berries in to component parts

About one hundred ripened berries from the identified infected and healthy plants of black pepper were collected in three replications and soaked in water overnight. The outer fleshy layer (mesocarp) was then removed by gentle crushing and the berries were thoroughly washed in sterile distilled water. The remaining part of the berry included outer perisperm, endosperm and embryo. Endosperm along with embryo was scooped out and the perisperm was separated from each berry followed by separation of embryo from endosperm under Leica-58APO stereo microscope. The separated parts (embryo, endosperm and perisperm) were stored at -20 ºC till DNA isolation.

Raising of seedlings

Berries of infected Panniyur-1, IISR-Thevam, Subhakara, and healthy plants were sown in sterile soil. Germinated seedlings were transplanted individually in to separate planting bags at four leaf stage and were kept in insect proof glass house for observation and to test by PCR for the presence or absence of PYMoV.

Collection of anthers

Anthers of black pepper flowers were removed from the spike with the help of a needle and transferred to 70% ethanol in a microfuge tube. About 10 mg of sample was collected from each of the three areas.

DNA extraction

Total genomic DNA isolation was performed from the leaves of seedlings, separated parts of berries and the anthers. Ten mg each of sample from parts of berries (embryo, endosperm and perisperm-three replications from each variety) and anthers from three different areas and 50 mg of leaf tissue from seedlings of all the four varieties were taken for total DNA isolation using the modified CTAB method as described by Hareesh and Bhat (2008). DNA from parts of berries and anthers were dissolved 10 µL while leaf DNA was dissolved in 50 µL of sterile nuclease free distilled water. Quality of DNA was checked through electrophoresis on 0.8% agarose and quantification was done in a Spectrophotometer (Eppendorf Biophotometer plus).

PCR detection of virus

PCR was performed using four different combinations of PYMoV specific primers (Table 1) amplifying different genomic regions of PYMoV to detect the presence or absence of virus in each seed parts and anthers. Total DNA isolated from each sample were tested initially by PCR with PYMoV specific primers AIB 35 and AIB 36 and the results obtained were further confirmed by PCR using three different PYMoV specific primer combinations- AIB 36/AIB 107, AIB104/AIB 105

Table 1. Details of primers used for amplification of different genomic regions of *Piper yellow mottle virus* by PCR

Primer name	Sequence (5'- - -3')	Orientation	Region amplified	Annealing Temp. (ºC)	Expected product size
AIB 35	TAACAGGACTAGGGATCG	Forward	ORF-I	52	480 bp
AIB 36	CAGCTGGTCTTGATAATAG	Reverse			
AIB 107	TATGCCAAGGTAAGCCCAAC	Forward	ORF III	52	900 bp
AIB 36	CAGCTGGTCTTGATAATAG	Reverse			
AIB 35	TAACAGGACTAGGGATCG	Forward	ORF I/III	52	2000 bp
AIB 159	TGACCTCATCAGTTGTTTC	Reverse			
AIB 104	CTATATGAATGGCTAGTGATG	Forward	ORF-III	56	350 bp
AIB 105	TTCCTAGGTTTGGTATGTATG	Reverse			

and AIB 35/AIB159. PCR reaction contained 1x PCR buffer (with $MgCl_2$), 100 µM dNTPs, 10 pM each forward and reverse primers, 2.5 U *Taq* polymerase, 2 µL template DNA and water to a final volume of 25 µL. The thermocycler was programmed for initial denaturation at 94 ºC for 5 min, followed by 35 cycles of denaturation at 94 ºC for 30 s, annealing at 52 ºC for 1 min, synthesis at 72 ºC for 1-2 min (depending on the primer), and a final extension for 10 min at 72 ºC. PCR products were analyzed by electrophoresis on 0.8% agarose gel.

Results and discussion

Identification of source plants

From the five symptomatic plants tested all Panniyur-1 and IISR-Thevam and three Subhakara plants were positive for PYMoV infection and gave an expected band of about ~480 bp on PCR with AIB 35/AIB 36 primers whereas all the five symptomless plants were found to be healthy and gave no amplification in PCR (Fig. 1). Three infected plants from each variety and one healthy plant were further used for the study.

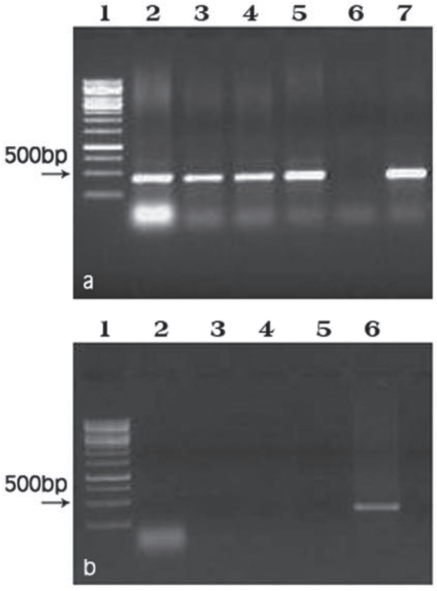

Fig. 1. Identification of PYMoV infected (a) and healthy (b) black pepper plants by PCR using virus specific primers. (a) Lane 1: 1 kb ladder, lane 2-5: PYMoV infected Panniyur-1 plants. Lane 6: Negative control, Lane 7: Positive control. (b) Lane 1: 1 kb ladder, lane 2-4: Healthy plants. . Lane 5: Negative control, Lane 6: Positive control. PCR was performed using primers AIB 35 and AIB 36, the details of which are given in Table 1.

Detection of PYMoV in different parts of berries and pollen grains by PCR

Good quality DNA was isolated using modified CTAB method. An average yield of 25 ng μL^{-1} DNA with 260/280 ratio of 1.8 was obtained from the samples. Results of PCR indicated presence of PYMoV on all parts of berries (embryo, endosperm and perisperm) in the three replications from the varieties tested, while it was absent in healthy plants. Anthers also gave expected band in PCR indicating the presence of PYMoV. Presence of virus was detected first by PCR with PYMoV specific primers AIB 35 and AIB 36 in berries (Fig. 2) and anthers (Fig. 3). These results were confirmed by PCR with three different combinations of PYMoV specific primers- AIB 36/AIB 107,

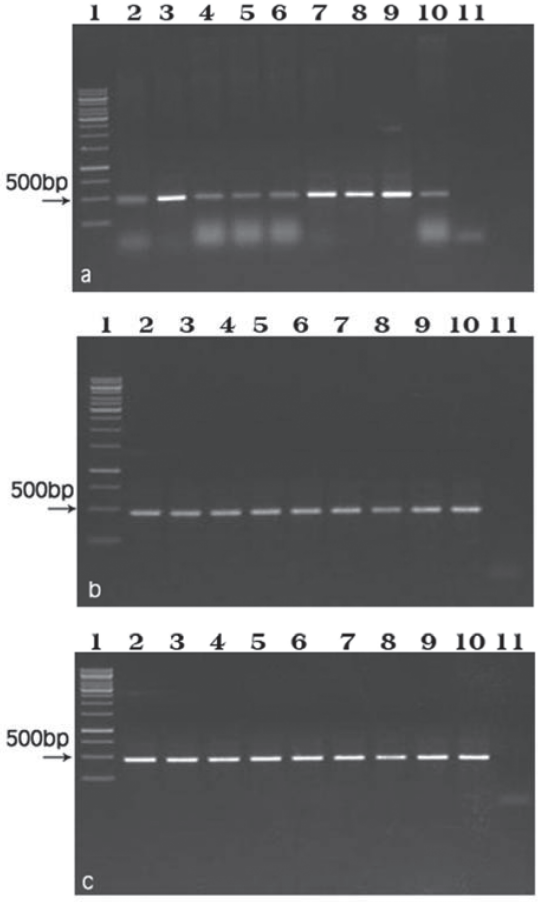

Fig. 2. Detection of PYMoV in different parts of black pepper berries by PCR. (a) Embryo (b) Endosperm (c) Perisperm. Lane 1: 1 kb ladder, lane 2-4: Panniyur-1, lane 5-7: IISR-Thevam, lane 8-10: Subhakara and lane 11: healthy control. PCR was carried out using primers AIB 35 and AIB 36.

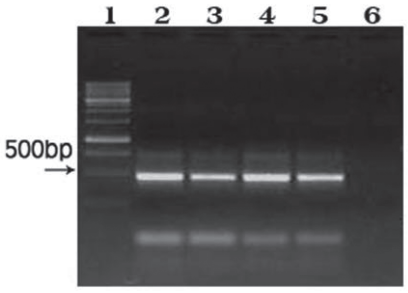

Fig. 3. Detection of PYMoV in black pepper anthers by PCR. Lane 1: 1 kb ladder, lane 2-4: Anther samples from three different areas. Lane 5: Positive control, Lane 6: Negative control. PCR was carried out using primers AIB 35 and AIB 36.

AIB104/AIB 105 and AIB 35/AIB159 and all of them gave positive results for the presence of PYMoV (Fig. 4). Identity of the PCR product was also confirmed by directly sequencing the gel eluted DNA band which matched with PYMoV sequence available at the GenBank (DQ836232 and DQ836226).

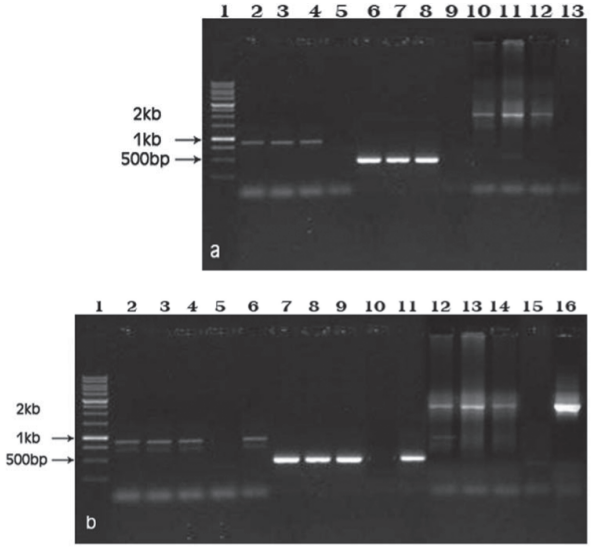

Fig. 4. Confirmation of the presence PYMoV in different parts of black pepper berries (a) and anthers (b) by PCR with different combinations of PYMoV specific primers. (a) Lane 1: 1 kb ladder, lane 2-4, 6-8 and 10-12: embryo, endosperm and perisperm of infected Panniyur-1 amplified with AIB 36 /AIB 107, AIB 104/AIB 105 and AIB 35 /AIB 159 respectively. Lane 5, 9 and 13: respective healthy control. (b) Lane 1: 1 kb ladder, lane 2-4, 7-9 and 12-14: Panniyur-1 samples from three different areas amplified with AIB 36 /AIB 107, AIB 104/AIB 105, AIB 35 /AIB 159 respectively. Lane 5-6, 10-11 and 15-16: respective healthy and positive control.

Detection of PYMoV in seedlings

Seedlings from berries of infected plants were positive for the virus infection in PCR while seedlings from berries of healthy plants were negative for virus infection (Fig. 5). Infected seedlings started showing yellow mosaic symptoms after two months of germination.

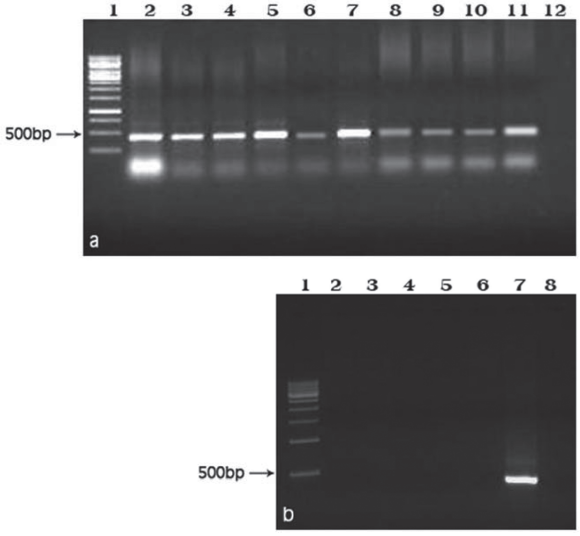

Fig. 5. Testing seedlings of black pepper for the presence of PYMoV by PCR. (a) Lane 1: 1 kb ladder, lane 2-10: seedlings from infected berries. Lane 11: positive control, Lane 12: Healthy control. (b) Lane 1: 1 kb ladder, lane 2-6: seedlings from healthy berries. Lane 7: positive control, Lane 8: Healthy control.

The study confirmed the presence of PYMoV in anthers and all the three parts of the berries-embryo, endosperm and perisperm. Seed transmission of PYMoV was reported (Hareesh and Bhat, 2010), but the present study confirmed the occurrence of virus in all the component parts of black pepper berries. This is the first information about the presence of PYMoV in anthers of black pepper. Seed transmission of other badnaviruses like *Kalanchoe top spotting virus* (KTSV), *Commelina yellow mottle virus* (ComYMoV*), Banana streak virus* (BSV), *Taro bacilliform virus* (TaBV) and *Cacao swollen shoot virus* (CSSV) were reported with different rates of transmission (Hearon and Locker, 1984; Medberry *et al.,* 1990; Daniels *et al.,* 1995; Macanawai *et al.,* 2005; Quainoo *et al.,* 2008). Seed transmission studies in CSSV showed the presence of virus in testa, cotyledon and embryo.

All the seedlings from infected seeds were also found to be positive for CSSV infection (Quainoo *et al*., 2008). TaBV was found to be seed transmitted in both self and cross pollinated taro plants (Macanawai *et al.,* 2005).

The present study also confirmed the presence of PYMoV in anthers of black pepper suggesting the possibility of the virus to be transmitted through pollen grains during fertilization. Badnaviruses such as CSSV, TaBV and KSTV (Hearon and Locke 1984; Macanawai *et al.,* 2005; Quainoo *et al.,* 2008) were also found to be present in pollen grains of their respective host plants. In general, viruses invade different seed parts, but seed transmission results most frequently from embryo infection (de Assis Filho and Sherwood, 2000). Presence of PYMoV in different parts of black pepper berries including embryo and its presence in seedlings raised from the infected berries clearly indicate occurrence of true seed transmission of PYMoV in black pepper. As black pepper is largely propagated by stem cuttings, seed transmission of PYMoV may not have much epidemiological significance. However, in breeding experiments it is important to use healthy mother plants since infected embryos and pollens can be a carrier of virus to the new progeny.

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