Research Article

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Seed transmission of *Piper yellow mottle virus* in black pepper (*Piper nigrum* L)

P.S. Hareesh and A.I. Bhat*

Division of Crop Protection, Indian Institute of Spices Research, Marikunnu P.O, Calicut-673012, Kerala, India

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Abstract

The study demonstrated the occurrence of true seed transmission of *Piper yellow mottle virus* (PYMoV) in black pepper (*Piper nigrum*) based on symptoms and polymerase chain reaction (PCR) tests of seedlings. The presence of PYMoV virions in berries and seedlings were confirmed by immunocapture (IC) PCR. This is the first report of occurrence of seed transmission of PYMoV.

Keywords: Black pepper, IC-PCR, PCR, *Piper yellow mottle virus*, seed transmission, symptoms

Introduction

Black pepper (*Piper nigrum* L) is one of the major spices grown in India and many South East Asian countries. Black pepper of commerce is a mature dried fruit (berry), valued for its characteristic pungency, aroma and flavor. *Piper yellow mottle virus* (PYMoV) (genus: *Badnavirus*) is known to infect black pepper in Brazil, Indonesia, Malaysia, Thailand, Philippines, Sri Lanka and India ((Lockhart *et al*., 1997; de Silva *et al*., 2002; Hareesh and Bhat, 2008). The virus induces chlorotic mottling, vein clearing, leaf distortion, reduced plant vigor and poor fruit set in affected black pepper plants. The virus is known to be transmitted primarily through vegetative means (stem cuttings) while secondary spread occur in field through various species of mealybugs (Lockhart *et al*., 1997; de Silva *et al*., 2002; Bhat *et al*., 2005). In addition, a few badnaviruses such as *Commelina yellow mottle virus* (ComYMV*), Kalanchoe top spotting virus* (KTSV) and *Cacao swollen shoot virus* (CSSV) are also known to be transmitted through seeds (Hearon and Locker, 1984; Medberry *et al*., 1990; Quainoo *et al*., 2008). Initial results on seed transmission of PYMoV in black pepper in Sri Lanka were negative (de Silva *et al*., 2002). In the present study we report the occurrence of true seed transmission of PYMoV in black pepper based on symptoms and PCR tests.

Materials and Methods

Seeds collected from PYMoV infected plant (identified by PCR) of four varieties (IISR-Sreekara IISR-Subhakara, IISR-Shakthi and Panniyur-1) were sown in separate seed pans in the insect proof glass house. Seeds collected from healthy plants (PCR negative plants) were also sown to serve as controls. Seedlings were planted in separate polythene bags after three months of germination and kept under insect proof condition for symptom development. For symptom observation, all the available seedlings were used.

To detect the presence of PYMoV in the berries and seedlings, PCR test (based on total DNA as template) was used. Total DNA was isolated from tissues using the protocol of Hareesh and Bhat (2008). Isolated total DNA was subjected to PCR using primers specific for open reading frame (ORF) I / ORF III region of PYMoV (Table 1). The PCR reaction (50 µl) contained 50 ng each of the primers, 1.5 U *Taq* DNA polymerase (Genei, Bangalore, India), 1x PCR buffer (Genei, Bangalore, India), 250 µM $MgCl_2$ and 10 µM each of the dNTPs (Finnzymes OY, Finland) and 31.5 µl of sterile water. PCR mix (45 µl) containing the above components was added to the tubes containing the template DNA (5 µl) resulting in a final reaction volume of 50 µl. The

*Corresponding author (E-mail: ishwarabhat@spices.res.in; aib65@yahoo.co.in)

Table 1. Details of primers used for the amplification of *Piper yellow mottle virus*

Primer name	Sequence (5′ .....3′)	Region	Annealing temp (°C)	Expected product size (bp)	Reference
SCBV-R1	CTCCTTCATCTCCTCAAGAAGCCT	ORF I	56	700	de Silva *et al*, (2002)
Badna 1R	CCAAAGCTCTGATAGCAGAC	ORF I			
Badna 3F 2	CTNTAYGAATGGYTWGTDATGCC	ORF III	58	600	Hareesh and Bhat, (2008)
Badna 3R1	CCAYTTRCAKAYKSCHCCCC	ORF III			
AIB 35	TAACAGGACTAGGGATCG	ORF I	50	450	Bhat and Siju, (2007)
AIB 36	CAGCTGGTCTTGATAATAG	ORF I			

temperature profile for the reaction involved the initial denaturation at 94 °C for 5 min followed by 35 cyles of denaturation at 94 °C for 30 s, annealing at 50/56/58 °C (depending on the primers used, see Table 1) for 1 min, synthesis at 72 °C for 1 min followed by final extension at 72 °C for 10 min. During DNA isolation and PCR, two controls were always used along with the test samples. These control samples included, a healthy virus free sample (negative control) and a known PYMoV infected sample (positive control). The PCR results were analyzed on 1 % agarose gel. The samples positive for PYMoV was identified based on the presence of expected size fragment obtained in the reaction (see Table 1). Initially to confirm the identity of amplicons, the PCR products obtained using all the three sets of primers were purified and directly sequenced at the automated sequencing facility of Genei, Bangalore, India.

In order to find out the presence of PYMoV particles (virions) in berries and seedlings, they were subjected to immunocapture (IC) PCR. PYMoV specific polyclonal antibodies developed at Indian Institute of Spices Research, Calicut, India (Bhadramurthy *et al*., 2005) was used at 1:1000 dilution to capture the virus particles. A 200 µl of diluted antibody was dispensed into 0.2 ml PCR tubes and incubated in a moist chamber at 37 °C for 2.5 h. Tissues were ground using sterile mortar and pestle in antigen extraction buffer (20 mM Tris, 138 mM NaCl, 1 mM PVP, 3 mM NaN_3 and 0.05 % Tween 20) at a ratio of 1:5 (w/v)) at room temperature followed by centrifugation at 8000 rpm for 30 s. The supernatant (200 µl) was added to the PCR tube after discarding and washing of the antibody solution. The unbound antigens were washed off with PBS-T after 2.5 h of incubation. After washing, the tubes were added with PCR components and subjected to thermocycling using three sets of primers as indicated above. The PCR products were analyzed on 1 % agarose gel. Identity of the amplicons was confirmed by direct sequencing of the PCR product at the automated sequencing facility of Genei, Bangalore, India.

Results and Discussion

The results of grow out test showed the highest number of symptomatic seedlings in the variety IISR-Sreekara (26 %) while in other varieties it ranged from 10-13 % (Table 2). The major symptom observed in all the varieties was mosaic (Fig. 1a and 1b) and no variety specific symptoms were seen. Other symptoms observed were chlorotic specks, mottling and brittleness of leaves. When 50 seedlings (including both symptomatic and asymptomatic) of each of the varieties were subjected to PCR, the highest percentage of PYMoV infected seedlings were seen in the variety IISR-Sreekara while in other varieties, it ranged from 22-28 (Table 2; Fig. 2). Identity of PCR product was confirmed by sequencing. The sequence matched with PYMoV sequence deposited in the GenBank Acc No. DQ836226, DQ836228 (data not shown). Compared to the symptomatological observation, higher percentage of infected seedlings were seen by PCR test indicating symptomless nature of some of the infected seedlings. The results presented showed that PYMoV is seed borne in black pepper.

The above results showed that PYMoV can be transmitted through seeds in different varieties of black pepper. A few of the badnaviruses such as *Banana streak virus* (BSV) and *Dracaena mottle virus* (DrMV) are

Table 2. Symptoms and PCR test for the detection of *Piper yellow mottle virus* in seedlings of different varieties of black pepper

Variety	No. of seedlings	No. of symptomatic seedlings	% symptomatic seedlings	No. of seedlings tested	No. of PCR positive seedlings	% infected seedlings
IISR-Sreekara	88	23	26	50	15	30
IISR-Subhakara	140	18	13	50	12	24
Panniyur -1	215	21	10	50	14	28
IISR-Shakthi	57	6	11	50	11	22

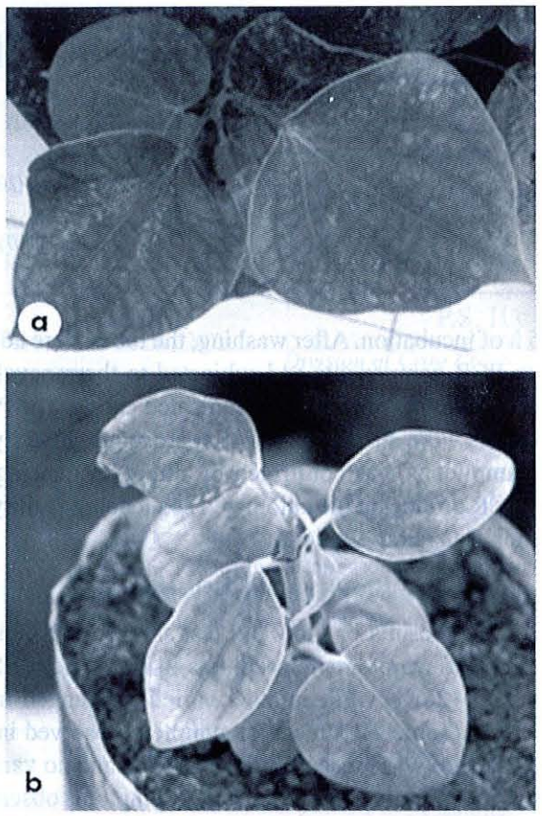

Fig. 1. Symptoms observed on black pepper seedlings raised from berries collected from *Piper yellow mottle virus* infected plant (a) mosaic (b) chlorotic specks

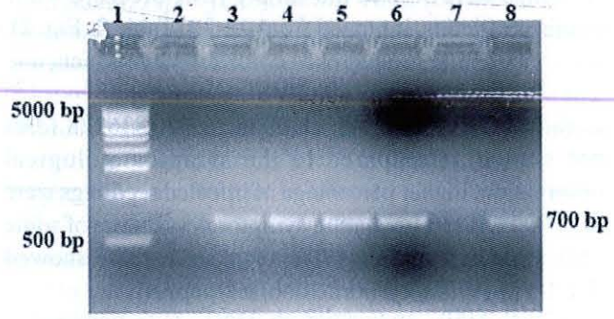

Fig. 2. Detection of *Piper yellow mottle virus* in seedlings by PCR using primers SCBV-R1 and Badna 1R. Lane 1: 500bp marker; Lanes 2-6: test seedlings; Lane 7: healthy (negative) control; Lane 8: known infected plant (positive control)

known to integrate their genome into the host genome. A PCR test can detect both integrated and episomal (virions) viruses while immunocapture (IC) PCR can detect only episomal viruses. Hence, in order to see whether PYMoV occurs in the form of virions in black pepper berries and seedlings, IC-PCR was carried out. The results of IC-PCR on berries and seedlings using three sets of primer pairs specific to PYMoV (Table 1) showed positive reaction clearly indicating the presence of PYMoV virions in berries and seedlings (Fig. 3a and b). The identity of the amplicon was confirmed through sequencing and comparison with PYMoV sequences from GenBank. These results clearly confirm the existence of true seed transmission of PYMoV in black pepper and hence, seeds can also serve as primary source of inoculum for the virus.

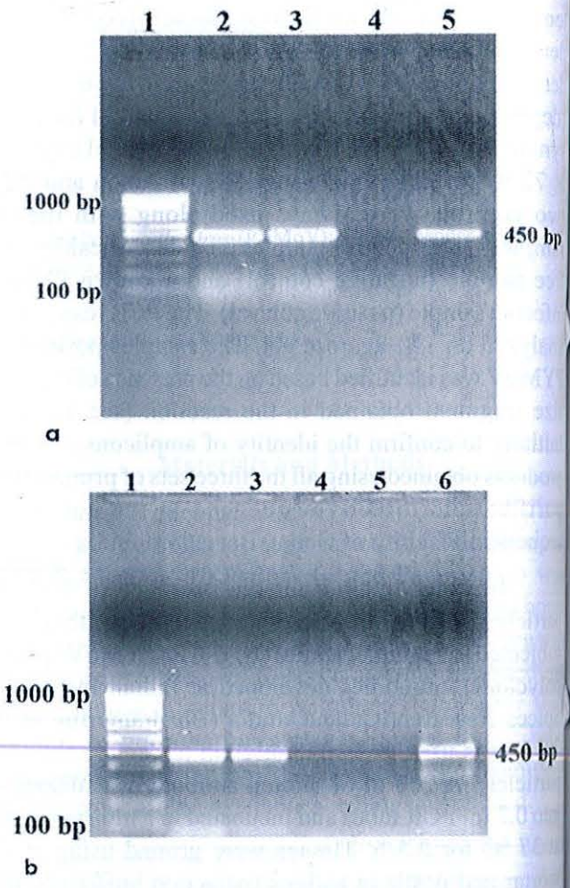

Fig. 3. Immunocapture PCR for detection of *Piper yellow mottle virus* in black pepper. (a) Detection in seedlings using primers, AIB 35 and AIB 36. Lane 1: 100 bp ladder; Lanes 2-3: test seedlings, Lane 4 healthy (negative) control; Lane 5: known infected plant (positive control). (b) Detection in berries using the primers, AIB 35 and 36. Lane 1: 100 bp ladder, Lane 2-4: test berries, Lane 5: healthy control (berry from healthy plant); Lane 6: positive control (berry from PYMoV infected plant)

Seed transmission was reported in a few *Badnavirus* species like KTSV (upto 40 %) (Hearon and Locker, 1984), ComYMV (upto 11 %) (Medberry *et al.*, 1990) and CSSV (Quainoo *et al.*, 2008). Different varieties of the same host species and even among different plants in a variety often vary widely in the rate at which seed transmission of a particular virus occurs

(Grogan and Bardin, 1950; Couch, 1955, Carroll and Chapman, 1970). In the present study, the variety IISR-Sreekara showed the highest percentage of infection both based on symptoms and PCR tests. Earlier studies of de Silva *et al.* (2002) reported non-transmission of PYMoV through black pepper seeds in Sri Lanka based on visual observation of symptoms and immunosorbent electron microscopy (ISEM) tests on seedlings raised from berries collected from infected black pepper plants. But results of the present work clearly demonstrated the occurrence of true seed transmission of PYMoV in black pepper based on symptoms and PCR tests. This is the first report of occurrence of seed transmission of PYMoV. Seed transmission may not have much significance in the spread of PYMoV in black pepper plantations as black pepper is mainly propagated through vegetative means. However, in breeding experiments this can cause considerable interference. The seed can be infected when one of the parents is infected as there is chance for the pollens and ovary to be infected. The present study also demonstrated the development and utility of IC-PCR in detecting PYMoV virions in plants and berries.

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