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Occurrence of symptomless source of *Piper yellow mottle virus* in black pepper (*Piper nigrum* L.) varieties and a wild Piper species

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Symptomless nature of *Piper yellow mottle virus* (PYMoV) infection in three varieties of black pepper (*Piper nigrum*) (Panniyur 1, Panniyur 5 and Panchami) and a wild species of *Piper* (*Piper colubrinum*) was confirmed by polymerase chain reaction (PCR) using PYMoV specific primers. The virus could be transmitted from these PYMoV-infected symptomless plants onto symptom producing black pepper cv. Karimunda through mealybug vector, *Ferrisia virgata* and by graft transmission. About 20–50% seedlings showed typical symptoms of the PYMoV at 30 days after mealybug inoculations while it was 75–94% at 90 days after inoculation. PCR test of the inoculated seedlings confirmed the presence of PYMoV in 50–64%, 76–100% and 80–100% of plants in 30, 60 and 90 days after inoculation, respectively. Similarly, 50–66%, 91–100% and 100% of graft-transmitted plants showed typical symptoms of the disease at 30, 60 and 90 days after grafting. PCR test of the graft-transmitted plants showed 100% PYMoV infection at 60 days after grafting. The results clearly demonstrated the existence of PYMoV-infected symptomless plants that can act as source for secondary spread of the virus in the field.

Keywords: Black pepper; *Piper nigrum*; *Piper yellow mottle virus*; symptomless plants; transmission; mealybug, graft

Introduction

Black pepper (*Piper nigrum* L.), the "king of spices" is native to Western Ghats of South India. The dried berries that are obtained from the vines have a high commercial value since they are being widely used as an important spice and condiment throughout the world. In India, the crop is mainly grown in Kerala and Karnataka. Nematodes, fungi and viruses are the major pathogens that affect black pepper plantations causing major loss to the black pepper industry (Ravindran 2000). *Piper yellow mottle virus* (PYMoV) (genus: *Badnavirus*) is an important virus affecting this crop worldwide (Lockhart et al. 1997; De Silva et al. 2002; Hareesh and Bhat 2008). Infected plants are characterised by chlorotic mottling, chlorosis, vein clearing, leaf distortion, stunted growth and poor fruit set. As black pepper is vegetatively propagated, the primary spread of the disease occurs through use of infected cuttings for planting and secondary spread in the field takes place through vectors such as mealybugs (*Ferrisia virgata, Planococcus citri* and *P. elisae*) and black

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pepper lace bug (*Diconocoris distanti*) (Lockhart et al. 1997; Duarte et al. 2001; De Silva et al. 2002; Bhat et al. 2003, 2005a, 2005b). Hence, identification of virus-free mother plants is important for propagation of healthy plants. Though external symptoms are good criteria for detection, sometimes depending on the season, growth stage and other factors, the disease can be difficult to identify or detect visually. Masking of symptoms during monsoon and winter months was seen in many of the affected black pepper vines (De Silva et al. 2002). Symptoms were best exhibited in the affected plants during March to May under Indian conditions (Bhat et al. 2005a, 2005b). Further, symptoms are exhibited prominently in certain cultivars like Karimunda, while symptoms are rarely seen in varieties such as Panniyur 1, Panniyur 5 and Panchami, and some of the plants in these varieties remain symptomless for many years. *Piper colubrinum* is a wild species known for its resistance against *Phytophthora capsici*, the causal fungus of foot rot disease. *P. capsici* is mainly soil borne and infects underground and collar region of plant (Anandaraj 2000). Hence, grafting black pepper on *P. colubrinum* root stock is followed to manage the disease. Though viral-like symptoms are not seen *in P. colubrinum*, black pepper plants grafted on them showed viral-like symptoms. Hence, present study was undertaken to study the existence of symptomless nature of PYMoV in three varieties of black pepper and *P. colubrinum* which can act as source for secondary spread of the virus in field.

Materials and methods

Identification of PYMoV infected symptomless plants

Symptomless plants of three varieties s of black pepper (Panniyur 1, Panniyur 5 and Panchami) and *P. colubrinum* collected from Indian Institute of Spices Research, Experimental Farm, Peruvannamuzhi, were subjected to polymerase chain reaction (PCR) using PYMoV specific primers as described by Bhat et al. (2009). Known positive and negative controls were kept in PCR. The identified PCR positive plants were then kept in glass house and used as source plants for transmission of the virus using mealybug and grafting.

Raising healthy plants

Healthy black pepper seedlings raised from seeds of cv. Karimunda under insect proof green house were used in both mealybug and graft transmission experiments. As PYMoV is also known to be transmitted through seeds, healthy yielding plants of cv. Karimunda were identified using PCR test as indicated earlier. Seeds collected form such PYMoV-free plants were used to raise seedlings under insect-proof conditions.

Transmission by mealybug

Mealybug transmission studies were done using *F. virgata* which is known to transmit PYMoV (Bhat et al. 2003). Non-viruliferous *F. virgata* reared on mature pumpkins in the laboratory were used in transmission studies. Mealybugs were picked up individually with the help of camel hair brush and allowed for an overnight acquisition access period on leaves collected from identified PCR positive but symptomless black pepper varieties and *P. colubrinum* leaves (on the lower

surface) kept in Petri dish lined with moist filter paper and covered with black cloth. For inoculation, healthy black pepper seedlings raised from seeds of cv. Karimunda under insect proof green house were used. Ten mealybugs each were transferred to the 45-day-old healthy test seedlings at four-leaf stage kept in a cage covered with black cloth. After overnight inoculation access period, the plants were sprayed with insecticide (chlorpyriphos 0.1%) to kill the mealybugs and were then removed from the cage and kept for observation in the insect-proof green house. Non-viruliferous (healthy) mealybugs inoculated onto healthy seedlings served as negative control while a known PYMoV-infected plant was used as source plant for acquisition.

Transmission by grafting

PYMoV-infected symptomless plants of black pepper varieties and *P. colubrinum* were used as root stock while healthy plants of black pepper cv. Karimunda were used as scion. For graft transmission, scions were excised from healthy Karimunda and top cleft grafted to virus-infected black pepper varieties and *P. colubrinum*. The graft insertion portion was taped tightly with parafilm to prevent desiccation, and the grafted plant was protected from excessive evaporation by a polythene bag cover and kept in insect proof glass house. The cover was removed after 1 week and plants were kept for observation in the insect-proof glass house.

Visual observations and PCR test

Plants of both mealybug and graft transmitted were observed for visible symptoms at 30, 60 and 90 days after transmission. Each of the transmitted plants was also subjected to PCR test for confirming presence or absence of PYMoV infection as described by Bhat et al. (2009). Briefly, total DNA isolated from 100 mg of leaf tissue was used as template for PCR. The primer pair for PCR detection was designed based on the ORF III sequence of the virus infecting black pepper in India (GenBank accession No. DQ836227). The forward primer (5′CTATATGAATGGCTAGTGATG3′) and reverse primer (5′TTCCTAGGTTT GGTATGTATG 3′) represented portion of ORF-III region. The positive PCR reaction was identified by specific amplification obtained at 400 bp. The identity of the amplicon was confirmed by directly sequencing the 400 bp product in an automated sequencing facility at Genei, Bangalore. Each PCR always included a known positive (infected black pepper) and negative (healthy black pepper) control. As virus titre was found to vary in plants, each sample was subjected to PCR using two template volumes namely, 1.0 μl and 0.5 μl in a 25 μl reaction volume. The PCR reaction contained 1 × PCR buffer, 2.5 mM $MgCl_2$, 200 μM dNTPs, 25 ng each of forward and reverse primers, 1.5 units of *Taq* polymerase, template DNA and sterile water to a final volume of 25 μl. The thermal cycler was programmed for initial denaturation at 94°C for 3 min, followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min, synthesis at 72°C for 1 min and a final extension for 10 min at 72°C. The PCR products were analysed on 1% agarose gel. A sample was considered positive if it gave expected amplified product using either 1 μl and/or 0.5 μl template volume. Total DNA isolation and PCR tests were repeated for all plants that gave negative results.

Results and discussion

Of the 10 symptomless plants of Panniyur 1, Panniyur 5 and Panchami each tested, all the plants were positive for PYMoV. Similarly, of the 25 symptomless *P. colubrinum* plants tested, 16 were positive for PYMoV in PCR test (Figures 1 and 2).

Transmission by mealybug

The PYMoV positive but symptomless plants of three cultivars of black pepper varieties and *P. colubrinum* were used as source plants for transmission of PYMoV on to healthy plants of cv. Karimunda through mealybugs. In the case of black pepper varieties, 36–50% of the inoculated Karimunda showed visible symptoms of PYMoV infection within 30 days while 70–88% and 75–94% of plants of inoculated Karimunda showed symptoms at 60 and 90 days after inoculation, although the source plants remained symptomless (Table 1). Similarly when *P. colubrinum* was used as source plant, 20% of the inoculated Karimunda showed visible symptoms of PYMoV infection within 30 days while 68% and 88% of plants of inoculated Karimunda showed symptoms in 60 and 90 days after inoculation, although the source plants remained symptomless (Table 1). Type of symptoms produced included mosaic, mottling, chlorosis and vein clearing (Figure 3). The symptoms were prominent and similar to those observed under natural conditions after 90 days of inoculation. Presence or absence of PYMoV in these plants was also confirmed by PCR. Identity of PCR products was confirmed by directly sequencing the gel-eluted PCR fragment (not shown). About 80–100% inoculated Karimunda seedlings were positive in PCR for PYMoV after 90 days of inoculation (Table 1; Figure 4).

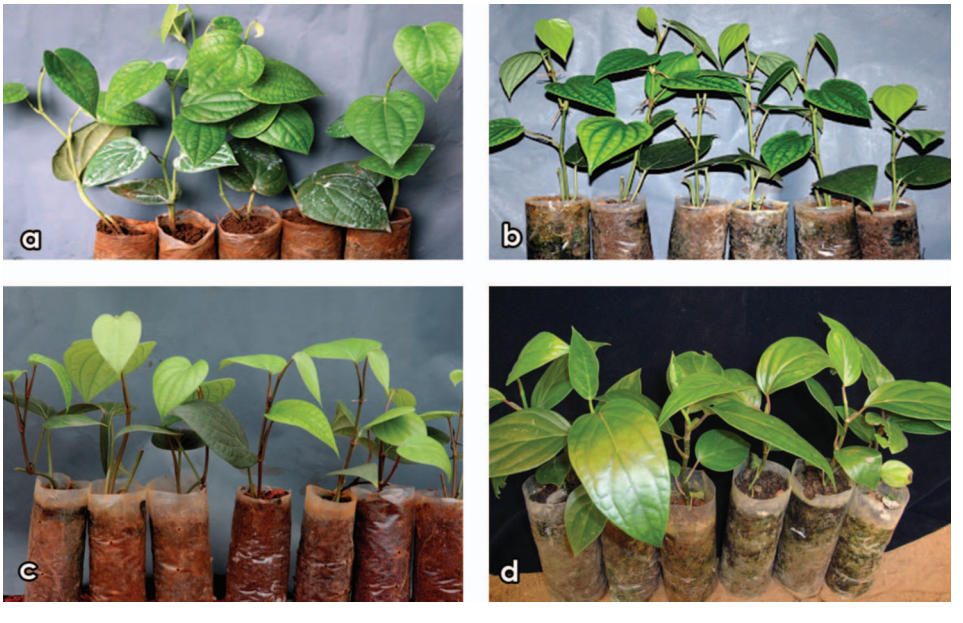

Figure 1. PYMoV-infected symptomless plants of black pepper varieties. (a) Panniyur 1, (b) Panniyur 5, (c) Panchami and (d) *Piper colubrinum*.

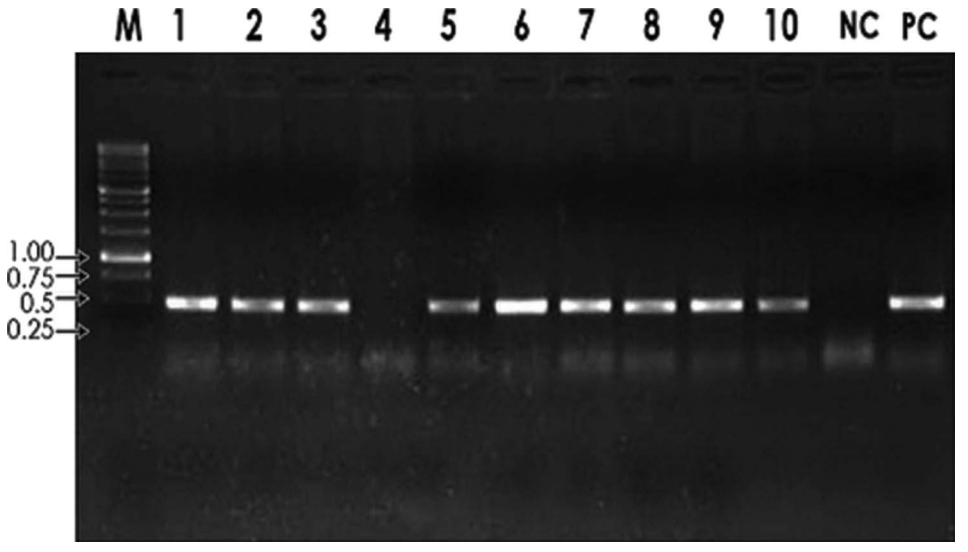

Figure 2. Polymerase chain reaction (PCR) test for the presence or absence or PYMoV in symptomless plants of black pepper varieties and *Piper colubrinum*. Lane M: Marker (1 Kb ladder); Lane 1–10: Field samples; Lane NC: Negative control; Lane PC: Positive control.

Transmission by grafting

The PYMoV positive but symptomless plants of three cultivars of black pepper and *P. colubrinum* were used as root stock and healthy plants of cv. Karimunda were used as scion for transmission of PYMoV through grafting, and 50–66%, 75–100% and 100% of grafted plants showed typical symptoms at 30, 60 and 90 days after grafting (Table 2; Figure 5). Presence or absence of PYMoV in these plants was also confirmed by PCR test. All the grafted plants tested positive for PYMoV at 60 days after grafting (Table 2; Figure 4).

Mealybug (*F. virgata*) was reported to transmit PYMoV from infected to healthy black pepper plants. In addition, being systemic in nature, PYMoV could also be transmitted experimentally by grafting from root stock to scion or vice versa (Bhat et al. 2003). In the present study, *F. virgata* could successfully transmit PYMoV from three varieties of black pepper and *P. colubrinum* to PYMoV-free healthy black pepper plants. Similarly, PYMoV could also be transmitted from infected but symptomless root stocks of black pepper and *P. colubrinum* to healthy scions by grafting. The transmission was judged based on the symptoms and later confirmed using more specific, reliable and sensitive techniques such as PCR. PCR test was reported to be more sensitive method for detection of viruses and is being used for detection and identification of PYMoV-free plants to be used for propagation (Bhat et al. 2009).

The results of the present study based on both mealybug and graft transmission tests clearly indicated that PYMoV-infected symptomless plants can act as source of PYMoV to other plants. Symptomless nature of a few badnaviruses such as *Banana streak virus* in banana (Lockhart 1995), *Citrus yellow mosaic virus* (CYMV) in citrus (Baranwal et al. 2003) and *Sugarcane mosaic virus* in sugarcane (Lockhart and Autrey 1988) are reported. However, so far there is no experimental evidence as to

Table 1. Transmission of PYMoV from symptomless plants of black pepper varieties and *Piper colubrinum* into symptom producing cv. Karimunda through mealybug.

Source plant variety	No. of plants with symptom/No. of plants inoculated			Type of symptoms	No. of plants positive in PCR/No. of plants tested		
	30 DAI	60 DAI	90 DAI		30 DAI	60 DAI	90 DAI
Panniyur 1	9/18 (50%)	16/18 (88%)	17/18 (94%)	MS, MT, CH,VC	10/18 (55%)	18/18 (100%)	18/18 (100%)
Panniyur 5	4/11 (36%)	8/11 (72%)	9/11 (81%)	MS, MT, CH,VC	6/11 (54%)	11/11 (100%)	11/11 (100%)
Panchami	8/20 (40%)	14/20 (70%)	15/20 (75%)	MS, MT, CH,VC	10/20 (50%)	20/20 (100%)	20/20 (100%)
P. colubrinum	5/25 (20%)	17/25 (68%)	22/25 (88%)	MS, MT, CH,VC	16/25 (64%)	19/25 (76%)	22/25 (80%)

Values given within parentheses indicate per cent of PYMoV infected plants; DAI, Days after inoculation; MS, Mosaic; MT, Mottling; CH, Chlorosis; VC, Vein clearing.

Figure 3. Symptoms observed on cv. Karimunda 60 days after transmission of PYMoV from symptomless plants through mealybug (Inset: close up showing symptoms).

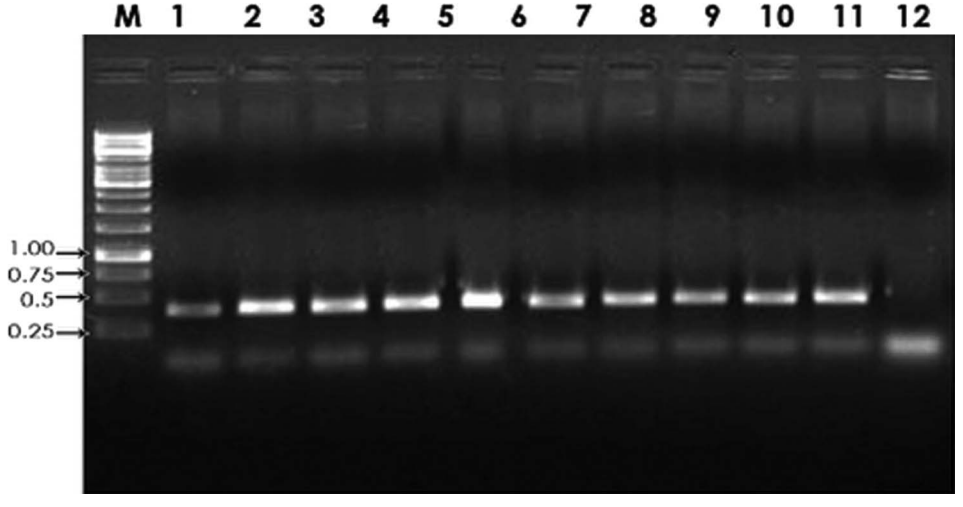

Figure 4. Polymerase chain reaction (PCR) test to confirm presence or absence of PYMoV in mealybug and graft transmitted black pepper cv. Karimunda. Lane M: marker; Lanes 1–10: mealybug and graft-transmitted samples; Lane 11: positive control; Lane 12: negative control.

whether such plants can be a source for secondary spread by insect vectors. Masking of symptoms depending on the season and growth stage of PYMoV infected black pepper plants has been reported from Sri Lanka and India (De Silva et al. 2002, Bhat et al. 2005a, 2005b). In addition, we have observed the presence of symptomless plants in certain varieties of black pepper which tested positive for virus infection

Table 2. Transmission of PYMoV from symptomless plants of black pepper varieties and *Piper colubrinum* into symptom producing cv. Karimunda through grafting.

Source plant variety	No. of plants with symptom/No. of plants inoculated			Type of symptoms	No. of plants positive in PCR/No. of plants tested		
	30 DAI	60 DAI	90 DAI		30 DAI	60 DAI	90 DAI
Panniyur 1	6/12 (50%)	11/12 (91%)	12/12 (100%)	MS,MT	12/12 (100%)	12/12 (100%)	12/12 (100%)
Panniyur 5	6/11 (55%)	9/11 (82%)	11/11 (100%)	MS,CH,CH	8/11 (73%)	11/11 (100%)	11/11 (100%)
Panchami	10/20 (50%)	15/20 (75%)	20/20 (100%)	MS,CH,CH	16/20 (80%)	20/20 (100%)	20/20 (100%)
P. colubrinum	12/18 (66%)	18/18 (100%)	18/18 (100%)	MS,CH,CH	14/18 (77%)	18/18 (100%)	18/18 (100%)

Values given within parentheses indicate per cent of PYMoV infected plants; DAI, Days after inoculation; MS, Mosaic; MT, Mottling; CH, Chlorosis; VC, Vein clearing.

Figure 5. Symptoms observed on cv. Karimunda used as scion 60 days after transmission of PYMoV from symptomless plants through graft (a) on *P. colubrinum* root stock (b) on Panniyur 1 root stock.

when tested by PCR. Since black pepper is vegetatively propagated through stem cuttings, it is important that mother plants for propagation is selected based on PCR test rather than only by visual symptoms. As *P. colubrinum* is used as root stock to combat *P. capsici*, it is also important to identify PYMoV-free plants for use as root stock in order to avoid PYMoV infection on the grafted plants.

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