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Polymerase chain reaction (PCR) based indexing of black pepper (*Piper nigrum* L.) plants against *Piper yellow mottle virus*

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Abstract

A polymerase chain reaction (PCR) based method was used for indexing 845 plants representing 14 popular varieties of black pepper (*Piper nigrum*) showing no visible external symptoms (apparently healthy) for *Piper yellow mottle virus* (PYMoV), among which 694 plants were positive (82%) for PYMoV. The percentage of infected plants ranged from 59% to 100% in different varieties; 100% of the tested plants of Panniyur-6 were infected with PYMoV and Sreekara had the least number of infected plants (59%). Some of the indexed PCR positive plants exhibited visible symptoms such as mild chlorosis, yellow specks and mottling in 1-3 months after testing. The study showed that indexing by PCR successfully detected PYMoV in infected black pepper plants showing no visible symptoms. The method can be used in certification programmes to identify PYMoV-free plants for further propagation.

Keywords: black pepper, indexing, PCR, *Piper nigrum*, *Piper yellow mottle virus*.

Abbreviations: CTAB=Cetyl trimethyl ammonium bromide; DNA=Deoxyribonucleic acid; dNTP=Deoxyribonucleotide triphosphate; EDTA=Ethylene diamine tetra-acetic acid; ELISA=Enzyme linked immunosorbent assay; ORF=Open reading frame; PVP=Polyvinyl pyrrolidone; PYMoV=*Piper yellow mottle virus*.

Piper yellow mottle virus (PYMoV), a Badnavirus, is an important virus associated with black pepper (*Piper nigrum* L.) in many countries such as Brazil, Indonesia, Malaysia, Philippines, Sri Lanka, Thailand and India (Lockhart *et al*. 1997; Duarte *et al*. 2001; de Silva *et al*. 2002; Bhat *et al*. 2003; Hareesh & Bhat 2008). Mosaic, mottling, reduction in leaf size and stunting of the plant are the major symptoms of the disease. The primary spread of the disease is by infected stem cuttings used as planting material while secondary spread in the field is through different species of mealybugs (Bhat 2008).

Proper detection of causal pathogens and use of healthy planting material are the prerequisites for integrated management of viral diseases. Seasonal variation, genotype, viral load, growth stage and other factors influence the expression of symptoms in a plant. Masking of visible external symptoms especially during monsoon months was observed in black pepper indicating that symptomatology cannot be the sole criterion

in identifying infected plants. ELISA based methods also failed to provide fool-proof detection of PYMoV owing to low titre of the virus in plants (Bhadramurthy *et al*. 2005). As black pepper is clonally propagated through stem cuttings, it is important to identify virus-free plants, to be used as mother plants for further propagation. Hence in the present study, a PCR-based method was developed and used in indexing black pepper plants, the results of which are presented here.

Fourteen popular varieties of black pepper plants collected from farmers fields and Indian Institute of Spices Research (IISR), Experimental Farm, Peruvannamuzhi (Kerala) were used in the study. The popular varieties included, Sreekara, Subhakara, IISR-Malabar Excel, IISR-Girimunda, IISR-Thevam, IISR-Shakthi, Panchami, Pournami, Panniyur-1, Panniyur-2, Panniyur-3, Panniyur-4, Panniyur-5 and Panniyur-6. The first fully opened leaf from each of the plants showing no visible symptoms (apparently healthy) were collected and processed for total DNA isolation.

Total DNA was isolated slightly modifying the protocol described by de Silva *et al*. (2002). Briefly, 100 mg of leaf tissue was ground in 500 µl of CTAB buffer [100 mM Tris HCl (pH 8), 4 mM EDTA (pH 8), 1.4 M NaCl, 2% CTAB, 1% PVP, 0.5% 2-mercaptoethanol] and incubated at 65°C for 30 min. The homogenate was allowed to cool to room temperature, extracted with an equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) and centrifuged at 2500 g for 10 min at room temperature. The supernatant was collected and 0.1 volume of 10% CTAB was added. This mixture was re-extracted using chloroform : isoamyl alcohol (24:1) followed by centrifugation at 2500 g for 10 min at room temperature. The supernatant was added with 0.1 volume of 3 M sodium acetate (pH 5.2) and an equal volume of isopropanol followed by incubation in ice for 30 min. The DNA was pelletized by centrifugation at 7000 g for 15 min at 4°C, washed in 70% ethanol, air dried and dissolved in 100 µl sterile distilled water.

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′ CTATATGAATGGCTAGTGATG 3′ and reverse primer 5′ TTCCTAGGTTTGGTATGTATG 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) controls. 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 x 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 analyzed on 1% agarose gel. A sample was considered positive if it gave expected amplified product using either 1.0 µl and / or 0.5 µl template volume. Total DNA isolation and PCR tests were repeated for all plants that gave negative results. The tested plants were kept under insect-proof conditions and observed for any visible symptom production.

In order to standardize the volume of template DNA required for the PCR, initially PCR was carried out using different template volumes such as 0.25 µl, 0.5 µl, 1.0 µl and 5.0 µl. Among them, 0.5 µl and 1.0 µl were found optimum. Direct sequencing of PCR

product showed 100% identity with PYMoV ORF III region sequence deposited at GenBank (DQ836227) thus confirming the specificity of the PCR product. Further, within 0.5 and 1.0 µl template volumes, 0.5 µl was found better as intensity of the band was higher (Fig. 1 a & b; Fig. 2 a & b). However, as infected field plants may contain varying concentrations of the virus, to score a plant either as positive or negative for PYMoV, each sample was subjected to PCR at two template volumes (0.5 µl and 1.0 µl) and the sample was considered positive if it gave expected amplified product either at 1.0 µl and /or 0.5 µl template volume.

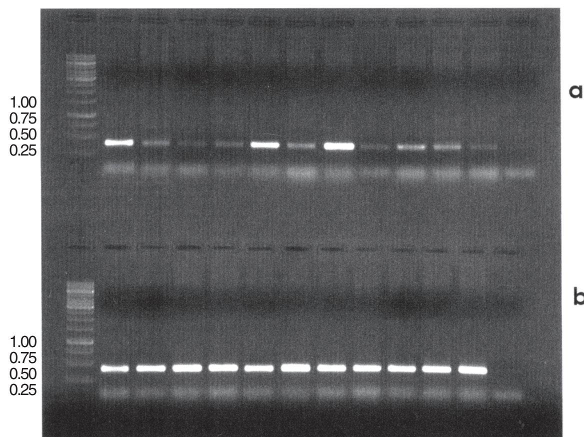

Fig. 1. Indexing black pepper plants for *Piper yellow mottle virus* (PYMoV) through PCR. Lane M: 1 Kb ladder; Lane 1: Positive control (known infected plant); Lanes 2-11: Test plants of var. Sreekara; Lane 12: Negative control (known healthy black pepper plant) (a) PCR performed with 1.0 µl template; (b) PCR performed with 0.5 µl template

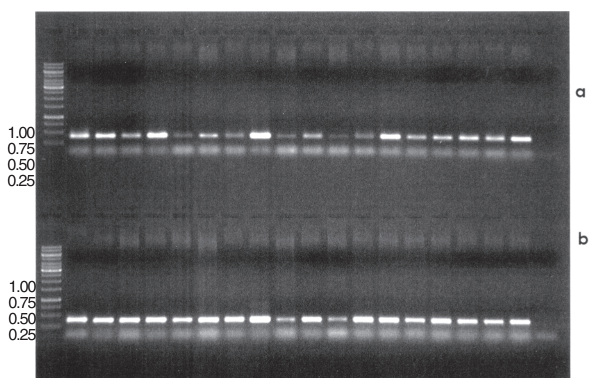

Fig. 2. Indexing black pepper plants for *Piper yellow mottle virus* (PYMoV) through PCR. Lane M: 1 Kb ladder; Lane 1: Positive control (known infected plant); Lanes 2-18: Test plants of var. Panchami; Lane 19: Negative control (known healthy black pepper plant) (a) PCR performed with 1.0 µl template; (b) PCR performed with 0.5 µl template

Among the 845 plants representing 14 varieties tested by PCR, 694 were positive for PYMoV (82%) indicating high incidence of the virus (Table 1; Figs. 1 and 2). The percentage of infected plants ranged from 59 to 100 in different varieties. Among the varieties, all the indexed plants of Panniyur-6 showed positive reaction (100%); Sreekara showed least number of infected plants (59%) (Table 1). Some of the indexed plants exhibited visible symptoms such as mild chlorosis, yellow specks and mottling in 1-3 months. The high incidence of the virus observed could be due to the use of cuttings from infected plant as source of planting material. Further, a few badnaviruses such as *Banana streak virus* and *Tobacco vein clearing virus* are known to integrate their genome into the host and such plants may remain symptomless (Harper *et al.* 1999; Lockhart *et al.* 2000). However, whether such a phenomena occur in PYMoV-black pepper system is yet to be studied.

The study showed that PCR method can be successfully used for the detection of PYMoV infection in black pepper especially in plants showing no visible symptoms. Variations in visible external symptoms from severe to complete absence of symptoms in infected black pepper plants were seen during different months of the year. This may be attributed to the influence of environmental factors on symptom expression. This kind of variation was reported for *Citrus yellow mosaic virus* infecting citrus, *Leek yellow stripe virus* infecting garlic and different viruses infecting potato (DeBokx & Piron 1977; Conci *et al.* 2002; Baranwal *et al.* 2003). When the symptoms are masked it is difficult to distinguish between healthy and infected plants. Masking of symptoms on black pepper plants infected with PYMoV makes it difficult to identify infected plants and hence sensitive

Table 1. Indexing black pepper nursery plants for the presence of PYMoV in different varieties through PCR

Variety	No. of plants tested	No. positive for PYMoV	Infected plants (%)
Sreekara	107	63	59
Subhakara	90	77	86
IISR-Thevam	58	43	74
IISR-Girimunda	43	33	77
IISR-Malabar Excel	76	59	78
Panchami	93	84	90
Pournami	47	39	83
IISR-Shakthi	64	52	81
Panniyur-1	106	104	98
Panniyur-2	23	20	87
Panniyur-3	67	58	86
Panniyur-4	11	9	82
Panniyur-5	22	15	68
Panniyur-6	38	38	100
Total	845	694	82

diagnosis is necessary to differentiate healthy and infected plants. ELISA-based method was not found to be fool proof always due to low titre of the virus in black pepper plants (Bhadramurthy *et al*. 2005). Many of the indexed PCR positive plants developed visible external symptoms with time. Similar results were also observed for *Apple stem grooving virus* in pear and apple certification program (Batlle *et al*. 2004). These results clearly indicate that a plant cannot be judged as healthy solely on the visible external symptoms but a much sensitive detection technique like PCR is useful in identifying healthy and infected black pepper plants. Thus the PCR method developed in the present study can be used in certification programmes to identify PYMoV-free plants for further propagation. The method would also be useful for screening germplasm to identify resistant sources against virus, detection in potential weed hosts and vectors and epidemiological studies.

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