DETECTION OF VIRUSES INFECTING BLACK PEPPER BY SYBR GREEN-BASED REAL-TIME PCR ASSAY

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SUMMARY

Virus infections are a major constraint to cultivation of black pepper (*Piper nigrum* L.), a perennial vegetatively propagated spice crop. Real-time PCR and real-time RT-PCR were developed to detect *Piper yellow mottle virus* (PYMoV) and *Cucumber mosaic virus* (CMV), respectively, in black pepper plants. These detection assays were up to 1000 times more sensitive than the conventional PCR/RT-PCR assays. Field samples from different pepper-growing regions in India were used to validate the two detection assays, which identified more infected plants than conventional PCR. Our results indicate that real-time PCR should be the method of choice for screening mother plants for PYMoV and CMV.

Key words: black pepper, *Cucumber mosaic virus*, *Piper yellow mottle virus*, PCR, RT-PCR, detection, sensitivity

INTRODUCTION

Black pepper (*Piper nigrum* L.), which originated in the tropical evergreen forests of the Western Ghats in India, is used for a variety of purposes including medicinal applications. Stunting of black pepper vines caused by viruses is becoming an increasingly serious problem because the systemic disease results in substantial yield reduction. The disease is characterized by mottled and smaller leaves, shorter internodes, stunted vines and has been reported from all major black pepper-growing countries in the world, i.e. Brazil, India, Indonesia, Malaysia, Philippines, Sri Lanka, Thailand, and Vietnam. *Cucumber mosaic virus* (CMV, genus *Cucumovirus*) and *Piper yellow mottle virus* (PYMoV, genus *Badnavirus*) are associated with the disease (Lockhart *et al*., 1997; Sarma *et al*., 2001; de Silva *et al*., 2002). CMV is a single-stranded RNA virus with a broad host range. The coat protein gene of a black pepper isolate of CMV showed more than 92% nucleotide sequence identity with CMV subgroup I. This virus isolate belongs to CMV subgroup IB (Bhat *et al*., 2005). PYMoV is a double-stranded DNA virus with a host range limited to *Piper* spp. and a close relationship with badnaviruses transmitted by mealybugs (Lockhart *et al*., 1997). A sequence identity higher than 95% was observed in a portion of the open reading frames (ORF) I and III of PYMoV isolates collected from different locations in India (Hareesh and Bhat, 2008). Both viruses have also been reported from other *Piper* species such as *Piper longum* L. (Indian long pepper) and *P. betle* L. (betelvine) (Hareesh *et al*., 2006; Siju *et al*., 2008).

Black pepper is vegetatively propagated from stem cuttings, a method that contributes to the dissemination of viruses, making the identification of virus-free mother plants even more critical for the management of virus diseases. Infected plants cannot be identified with certainty by visual symptoms because infected plants are not always symptomatic. The more sensitive and reliable methods currently available for the detection of PYMoV and CMV are PCR and RT-PCR, respectively, and combined multiplex RT-PCR (for both viruses) (de Silva *et al*., 2002; Bhat and Siju, 2007; Siju *et al*., 2007; Bhat *et al*., 2009). Real-time PCR, a highly sensitive method for plant virus detection (Mackay *et al*., 2002), combines the simplicity of PCR amplification with the sensitivity of fluorescence detection during amplification and does not require post-PCR processing. Thus, as reported in this paper, real-time PCR and real-time RT-PCR assays were finalized for PYMoV and CMV detection in black pepper.

MATERIALS AND METHODS

Virus isolates and nucleic acid isolation. For standardization of real-time PCR and real-time RT-PCR assays, samples from a healthy black pepper plant and four plants infected by PYMoV and CMV were collected from different geographical areas of Karnataka and Kerala, the two major black pepper-growing states of India. The virus status of these plants was ascertained by multiplex RT-PCR (Bhat and Siju, 2007). The same sources were used for determining virus detection limits and compare them to those of conventional PCR and RT-PCR. Materials used for validating the assays comprised 159 samples of black pepper (64 with and 95 without the characteristic symptoms), five samples of betelvine and 10 samples of Indian long pepper, all symptomatic (Table 1). Total nucleic acids (TNA) were isolated from black pepper plants following the procedure of Bhat and Siju (2007) and their yield was

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Table 1. Validation of real-time PCR and real-time RT-PCR for the detection of *Piper yellow mottle virus* (PYMoV) and *Cucumber mosaic virus* (CMV) in field samples of black pepper, Indian long pepper, and betelvine from different regions of India.

Sample	No. of samples tested		No. of positive samples in conventional PCR/ RT-PCR				No. of positive samples (range of Cq values) in real-time PCR and real-time RT-PCR			
	Symptoms	No symptoms	Symptoms		No symptoms		Symptoms		No symptoms	
			PYMoV	CMV	PYMoV	CMV	PYMoV	CMV	PYMoV	CMV
Black pepper	64	95	63	9	58	-	64 (14–33)	12 (9–30)	60 (15–32)	-
Betelvine	5	-	5	3	-	-	5 (11–24)	3 (11–26)	-	-
Indian long pepper	10	-	10	8	-	-	10 (13–23)	8 (11–24)	-	-

determined using a spectrophotometer (Biophotometer plus, Eppendorf, Germany).

Primer design and synthesis. Real-time PCR primers for PYMoV were designed based on conserved sequences in the 600 bp region of ORF III of five isolates for which information is available in GenBank (accession Nos. DQ836227, DQ836229, DQ836231, DQ836232, and DQ 836235) (Benson *et al*., 1999). The forward primer sequence comprised nucleotides (nt) 160 to 179 (PYMoV-F 5'-CACTTAGTCGCAATGCTGGA-3') and the reverse primer the sequence from nt 247 to 266 (PYMoV-R 5'-CCAATAGTTGCTCCCAGGAA-3') with an expected amplicon of 107 bp. Real-time RT-PCR primers for CMV were designed based on the conserved sequences of the 657 bp coat protein gene identified by multiple sequence alignment of 15 CMV subgroup IB isolates (including the black pepper isolate of CMV) retrieved from GenBank. The forward primer sequence comprised nt 362 to 381 (CMV-F 5'-TGTGGGTGACAGTCCGTAAA-3') and the reverse primer the sequence from nt 453 to 472 (CMV-R 5'-ACGCGGCATACTGATAAACC-3') with an expected amplicon of 111 bp. The specificity of the primers was checked using the BLASTn, and the secondary structure and intra- and inter-primer complementarity were checked using Oligo Calc (www.basic.northwestern.edu/biotools/oligocalc.html). The primers were synthesized at Integrated DNA Technologies (Coralville, Iowa, USA).

Optimization of real-time PCR and real-time RT-PCR. Both these assays were performed on a Rotor Gene Q real-time PCR system (Qiagen, Germany). Reaction components for real-time PCR (for PYMoV) and real-time RT-PCR (for CMV) were optimized using TNA extracted from infected (positive control) and healthy (negative control) plants. Water was used as an additional negative control. Real-time PCR reaction for PYMoV was carried out in a final volume of 25 µl containing 12.5 µl of 2× QuantiFast^{TM} SYBR Green PCR Master mix (Qiagen, USA), 1.0 µl each of the forward and reverse primers (final concentrations ranging from 50 nM to 500 nM were tested to determine the optimum concentration), and 1 µl of the template nucleic acid (about 100 ng). Thermocycling conditions consisted of an initial denaturation step at 95°C for 5 min followed by 35 cycles of 95°C for 15 sec, 56-60°C (to determine the optimal annealing temperature) for 20 sec, and 60°C for 20 sec. The component of real-time RT-PCR reaction for CMV consisted of 25 U of Revert Aid reverse transcriptase (Fermentas, Lithuania) in addition to the above components. Thermocycling conditions included one additional step, namely cDNA synthesis at 42°C for 45 min. Following real-time PCR and real-time RT-PCR, the amplicons were subjected to melt analysis from 60°C to 95°C. The specificity of products of the real-time PCR and real-time RT-PCR was also periodically verified by electrophoresis on a 1.5% agarose gel and by direct sequencing of the gel-purified PCR products.

Determination of detection limits for viruses and their comparison with those of conventional PCR. To compare the detection limits of different assays, TNA isolated from infected plants was diluted serially up to 10^{-5} using TNA from a healthy plant as a diluent. Each dilution (1 µl) was subjected to conventional and real-time PCR (for PYMoV) and to conventional and real-time RT-PCR (for CMV) carried out as described earlier (Siju *et al*., 2007; Bhat *et al*., 2009) using the same set of primers.

Validation of real-time PCR and real-time RT-PCR assay. In order to validate the detection of PYMoV and CMV in field samples, TNAs (DNA and RNA) isolated from all test samples were subjected to real-time PCR and real-time RT-PCR along with the positive and negative controls. Prior to this validation, these samples were subjected to conventional PCR and RT-PCR for the detection of PYMoV and CMV, respectively.

RESULTS

SYBR Green real-time PCR and real-time RT-PCR assay development. The SYBR Green-based real-time PCR and real-time RT-PCR amplified products from the four infected plants from four locations in Karnataka and Kerala for both viruses, whereas no amplification was obtained from both negative controls (Fig. 1a, b). The melt curve of real-time PCR and real-time RT-PCR products for positive control samples had a single peak at 82.5-84.0°C,

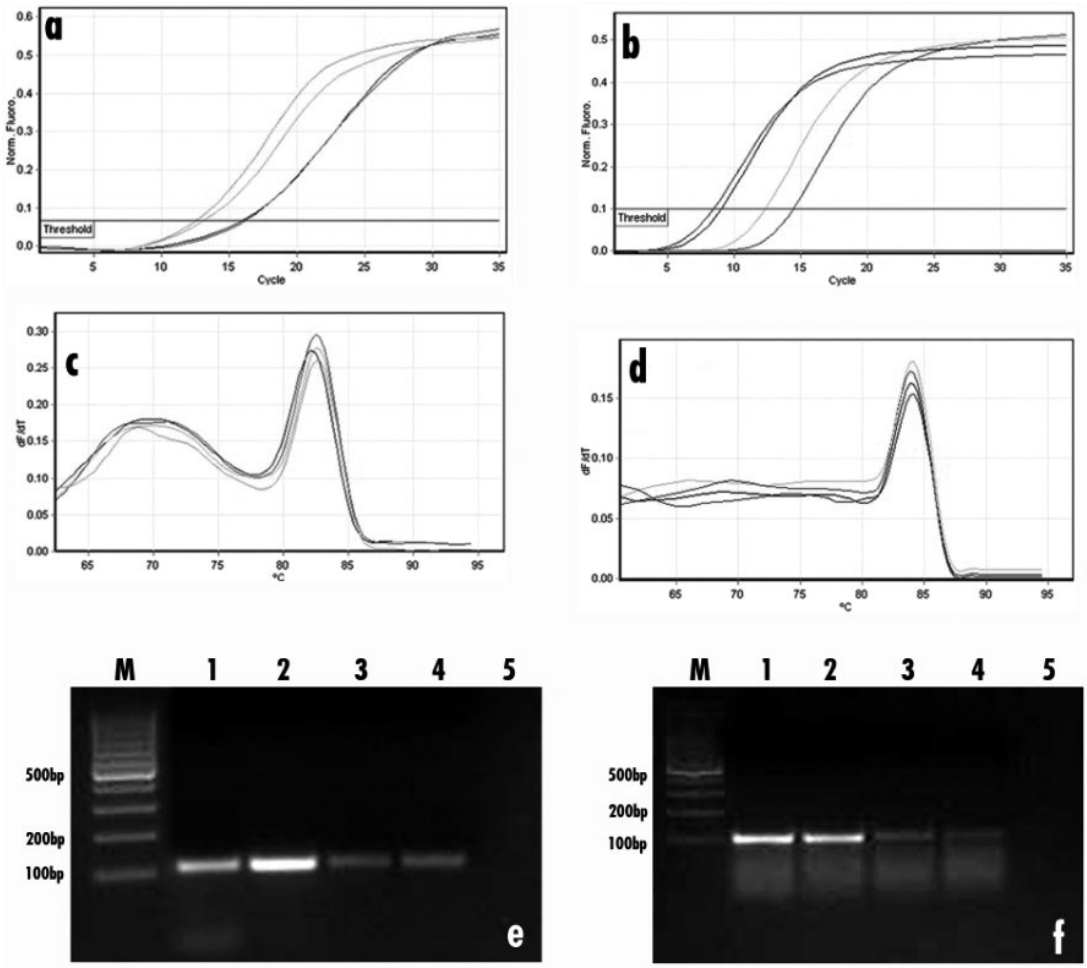

Fig. 1. Standardization of real-time PCR and real-time RT-PCR for the detection of *Piper yellow mottle virus* and *Cucumber mosaic virus* in black pepper: (a, b) amplification curves of real-time PCR and real-time RT-PCR obtained with positive controls; (c, d) melt curve analysis of real-time PCR and real-time RT-PCR products; (e, f) agarose gel electrophoresis of real-time PCR and real-time RT-PCR products; lane M, 100 bp DNA ladder; lanes 1-4, infected black pepper; lane 5, healthy black pepper. Amplification curve, melt curve, and the reaction products were not seen with healthy plants and the water control.

indicating that only the target fragment was amplified (Fig. 1c, d). Both negative controls showed no melting peak. The specificity of the product was confirmed by agarose gel electrophoresis with a single band of the expected size (Fig. 1e, f), and by sequencing. The product of real-time PCR showed 99% identity with the sequence of a portion of ORF III of PYMoV (GenBank accession No. DQ 836232), whereas the product of real-time RT-PCR showed 100% identity with the portion of the coat protein gene of a black pepper isolate of CMV (GenBank accession No. AY545924). The optimum concentration of the primers was 200 nM for both PYMoV and CMV and the optimum annealing temperature was 60°C for both PYMoV and CMV (data not shown).

Determination of detection limits for viruses and comparison with those of conventional PCR. The detection limit for PYMoV using real-time PCR was 10^{-3} whereas conventional PCR could detect the virus only in undiluted samples (Fig. 2a, c). The detection limit for CMV using real-time RT-PCR was 10^{-4} and that using conventional RT-PCR was 10^{-1} (Fig. 2b, d). Thus both real-time PCR and real-time RT-PCR were up to 1000 times more sensitive than conventional PCR and RT-PCR assays.

Validation of real-time PCR and real-time RT-PCR assays. When tested by using conventional PCR and RT-PCR, nine of the 64 samples from vines with characteristic disease symptoms tested positive for both PYMoV and CMV, whereas all the remaining samples (except one) tested positive only for PYMoV (Table 1). The results of 64 samples subjected to real-time PCR and real-time RT-PCR assays showed 12 samples positive for both PYMoV and CMV; all the remaining samples were positive only for PYMoV. Of the 95 symptomless black pepper samples that were subjected to conventional PCR and RT-PCR, 58 tested positive for PYMoV and none for CMV. When these 95 samples were subjected to real-time PCR and real-time RT-PCR, 60 (including the 58 that had tested positive for PYMoV in conventional PCR) tested positive for PYMoV and none for CMV. All the samples of Indian long pepper and betel vine plants exhibiting characteristic disease

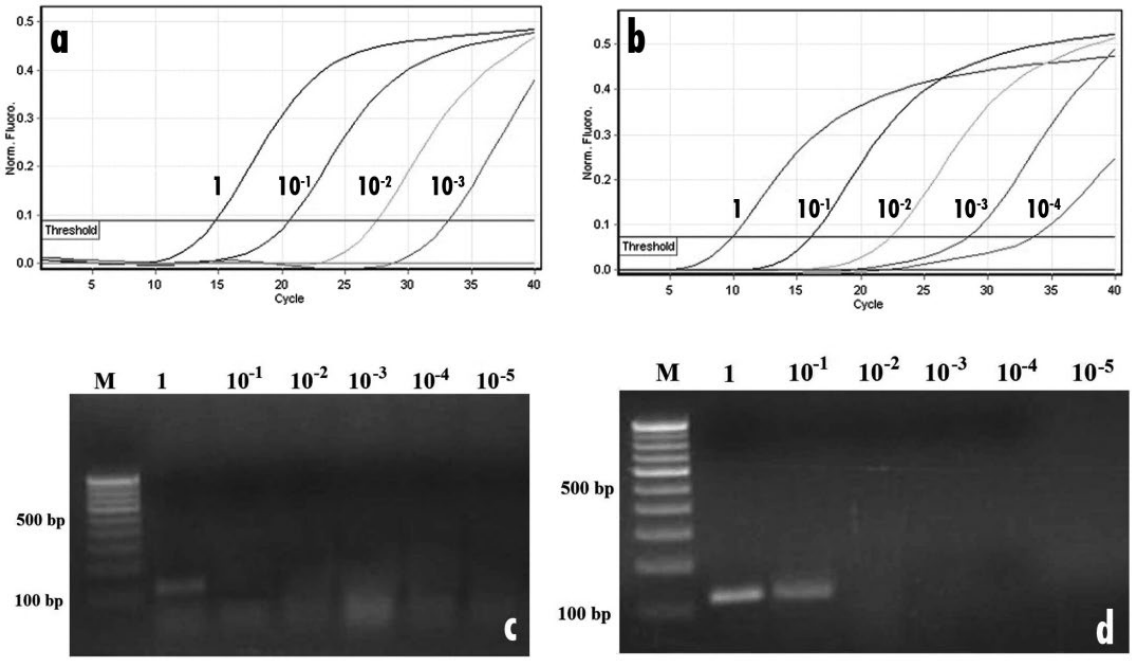

Fig. 2. Comparison of detection limits of *Piper yellow mottle virus* (PYMoV) and *Cucumber mosaic virus* (CMV) in black pepper by conventional and real-time PCR: (a), real-time PCR for the detection of PYMoV; (b), real-time RT-PCR for the detection of CMV; (c) conventional PCR for the detection of PYMoV; and (d) conventional RT-PCR for the detection of CMV. Lanes 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} show different dilutions of the total nucleic acids; lane M shows a 100 bp DNA ladder.

symptoms yielded similar results in both conventional PCR/RT-PCR and real-time PCR/RT-PCR. The quantification cycle (Cq) values of the positive samples subjected to real-time PCR varied from 14 to 33 for black pepper, 11 to 24 for betelvine, and 13 to 23 for Indian long pepper, and those subjected to real-time RT-PCR varied from 9 to 30 for black pepper, 11 to 26 for betelvine, and 11 to 24 for Indian long pepper (Table 1). There was no correlation between symptoms and Cq values. All the plants that were infected with PYMoV and CMV were stunted and showed mottling, deformation, small leaves and short internodes, whereas the symptoms varied a great deal in those infected with PYMoV alone: some plants were symptomless while others exhibited mild to severe mottling, deformation of the leaves, and reduction in leaf size.

DISCUSSION

Compared to conventional PCR and RT-PCR, real-time PCR and real-time RT-PCR assays are not only more sensitive but also less time consuming. These detection assays are routinely used for the detection of various pathogens including viruses. In the present study, SYBR Green-based real-time PCR and real-time RT-PCR assays were standardized for the detection of PYMoV and CMV, respectively, in black pepper. CMV has been detected in banana (Lili *et al*., 2009), lily (Kouassi *et al*., 2010), dormant bulbs (Lim *et al*., 2010), gerbera (Wei *et al*., 2012) and black pepper (this study) by real-time RT-PCR. To our knowledge, this is the first report of a real-time PCR assay for PYMoV.

The real-time PCR and real-time RT-PCR assays could detect PYMoV and CMV in samples collected from different black pepper-growing regions of India, indicating a successful design of primers and optimization of the assays. The specificity of the reaction was shown by a consistent melt peak at 82.5-84°C, lack of cross-reactivity between CMV and PYMoV infecting black pepper, identification of an amplicon as a single band of the expected size by agarose gel electrophoresis, and the absence of amplification signals in negative controls (healthy plants and water). The specificity of the CMV and PYMoV amplicons was confirmed by sequencing.

With respect to detection limits, our results are consistent with those obtained by others (Olmos *et al*., 2005; Loconsole *et al*., 2010; Shiller *et al*., 2010; Harper *et al*., 2011) with both types of assays which proved to be up to 1000 times more sensitive than conventional PCR and RT-PCR. The diagnostic ability of our assays was validated by testing field samples from different hosts and locations, proving suitable for the quick and reliable detection of CMV and PYMoV. However, since real-time PCR detected viruses in more plants than conventional PCR (3%, 6 of 174) it qualifies as the method of choice for screening mother plants.

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