Development of a single-tube multiplex RT–PCR for the simultaneous detection of *Cucumber mosaic virus* and *Piper yellow mottle virus* associated with stunt disease of black pepper

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Single-tube multiplex RT–PCR was developed for the simultaneous detection of *Cucumber mosaic virus* and *Piper yellow mottle virus* in black pepper. PCR primers designed for amplifying 650 bp from the coat protein gene of CMV and 450 bp from the open reading frame I of PYMoV were used in the reaction. The quantity of template used in mRT–PCR was found to be crucial for successful amplification. The method was found successful for the detection of both the viruses infecting black pepper in nursery and field samples. The method is rapid, reliable and requires only small tissue sample for sensitive detection. The entire procedure can aid in the rapid screening of a large number of plants for both viruses.

Keywords: Black pepper, *Cucumber mosaic virus*, *Piper yellow mottle virus*, total nucleic acid isolation.

BLACK pepper (*Piper nigrum* L., Piperaceae) is an economically important spice grown in many South East Asian countries, including India. High incidence and severity of stunt disease was reported in India from black pepper plantations located especially in high attitudes[1]. The association of two distinct viruses with the disease, viz. *Cucumber mosaic virus* (CMV, genus *Cucumovirus*; family Bromoviridae) and *Piper yellow mottle virus* (PYMoV, genus *Badnavirus*; family Caulimoviridae) was reported from Brazil, India, Indonesia and Sri Lanka[2–6]. However, association of PYMoV alone with the disease was reported from the Philippines and Thailand[7]. CMV is one of the most widespread RNA viruses of substantial agricultural importance, infecting more than 1000 plant species[8]. The genome of the virus consists of three positive sense, single-stranded RNAs (RNA 1–3) and a sub genomic RNA (RNA 4) which is involved in encapsidation[9]. Based on the nucleotide sequence homology and serological properties, numerous strains of CMV have been classified[9] into two major subgroups, I & II. Subgroup I has been further divided into two subgroups (IA and IB) based on phylogenetic analysis and gene sequences[10,11]. Based on the coat protein sequence homology, CMV infecting black pepper was identified[5] to be a strain belonging to subgroup IB. PYMoV is a bacilliform, monopartite virus with double-stranded DNA genome and has a narrow host range[7]. PYMoV infecting black pepper has been characterized from Sri Lanka and Thailand[6,7]. The major means of spread of CMV and PYMoV is through the use of infected stem-cuttings. Hence use of virus-free planting material is important to check the spread of the disease. Symptoms cannot be used as the sole criterion to identify healthy plants, as masking of symptoms during monsoon and winter months is seen in many of the affected vines. ELISA is not a reliable method especially for the detection of PYMoV, as it occurs at a low titre in plants[7,12].

PCR-based techniques are highly sensitive and are employed for the detection of plant viruses. Detection of viruses by PCR relies on the isolation of amplifiable/good quality DNA and RNA free from polyphenols, polysaccharides and endogenous nucleases. Phenolics are readily oxidized to form covalently linked quinones and bind with nucleic acids[13]. Endogenous nucleases reduce integrity of nucleic acids and thereby inhibit detection of nucleic acid targets in PCR. Multiplex PCR (m-PCR) is a variant of PCR in which two or more loci are simultaneously amplified using several pairs of primers in a single reaction. Therefore, it is considered as a rapid method for the detection of viruses in legumes[14], grapevine[15], olive trees[16], sugar beet[17], potato[18] and rice[19]. However, in all these cases (except in rice), detection of either many RNA or DNA (not RNA and DNA) viruses was reported. In this study, we evaluated the simultaneous isolation of amplifiable total nucleic acid from black pepper that aids in the development of a single-tube mRT–PCR for the detection of the RNA virus (CMV) and DNA virus (PYMoV) from the same sample.

The virus isolates were collected from black pepper vines of plantations from Kodagu District, Karnataka; and Kozhikode, Idukki and Wynad districts, Kerala, India. These isolates were maintained through vegetative propagation under controlled insect-proof glasshouse conditions at Indian Institute of Spices Research, Calicut. Tender, young leaf samples collected from symptomatic, asymptomatic, PCR-positive (CMV and PYMoV positive control) and healthy (negative control) plants were used in the study. Experiments were repeated at least two times to score a plant as PCR-positive or negative.

For extraction of the total nucleic acids (DNA and RNA), a modified protocol was developed by combining methods described earlier[20,21]. Briefly, 50 mg of tender black pepper leaf tissue from healthy and infected plants was ground separately in 500 µl of denaturing solution [4 M guanidium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% (w/v) sodium N-lauryl sarcosine, 0.1% (v/v) β-mercaptoethanol, 0.5% (w/v) sodium sulphite] and collected in a microcentrifuge tube. To this, 50 µl of 2 M sodium acetate (pH 4.0), 500 µl of water-saturated phenol and 100 µl of chloroform : isoamyl alcohol (49 : 1) were added serially with intermittent mixing at each step. The tube was then shaken for 15 s and kept on ice for 15 min. The resulting homogenate was centrifuged at 12,000 *g* for 15 min at 4°C, which

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resulted in the separation into three phases – aqueous phase, organic phase and interphase. The aqueous phase was carefully collected in a fresh tube and mixed with equal volume of isopropanol followed by incubation at –80°C. Next 0.5 volume of 0.1 M Tris (pH 10.5) was added to the organic phase and interphase, gently vortexed for 15 s, incubated on ice for 30 min followed by centrifugation at 2000 *g* for 15 min at 4°C. The resulting supernatant was extracted twice with equal volume of chloroform : isoamyl alcohol (24 : 1) followed by centrifugation at 12,000 *g* for 10 min at 4°C. To the final supernatant, 0.1 volume of 3 M sodium acetate (pH 4) and 1 volume of ice-cold isopropanol were added and mixed well by gentle pipetting. This entire sample was then added to the tube containing RNA kept at –80°C and incubated for a further 1 h for precipitation of total nucleic acids. Centrifugation at 12,000 *g* for 10 min at 4°C yielded a pellet, which was washed with 75% ethanol and air-dried. The final pellet was dissolved in 80.0 µl of sterile HPLC grade water.

Two pairs of compatible oligonucleotide primers suitable for single-tube mRT–PCR were designed on the basis of published sequence data of black pepper isolates of CMV (GenBank accession no. AY545924) and PYMoV (GenBank accession no. DQ836226). Sequences of oligonucleotides along with their expected amplicon size are listed in Table 1. Specificity of the primers was checked using BLAST[22] and compared with other sequenced isolates deposited in GenBank.

A single-tube mRT–PCR was set up for amplification of CMV and PYMoV. For initial standardization, mRT–PCR contained 1× PCR reaction buffer (Genei, Bangalore, India), 1.5 mM $MgCl_2$ (Genei), 10 mM DTT, 375 µM dNTPs (Merck, India), 10 U RNasin (Genei), 15 pmol each of the four primers, 1.5 U *Taq* DNA polymerase (Genei) and 20 U MuMLV RT (Finnzymes, OY, Finland). A range of template volumes was used in separate reactions and the final volume was made up to 50.0 µl by adding sterile HPLC-grade water. The finally adopted mRT–PCR contained half the volume of all the above reagents. Separate PCR and RT–PCR containing individual sets of specific primers were set up along with the multiplex reaction for comparison. Synthesis of cDNA was performed at 42°C for 45 min and amplification was carried out for 40 cycles in an automated thermocycler (Eppendorf master cycler, gradient). Each cycle consisted of denaturation at 94°C (30 s), primer annealing at 50°C (1 min) and extension at 72°C (1 min), followed by a final extension at 72°C (10 min).

Amplified products (25 µl of 50 µl reaction mix) were analysed with 500 bp DNA ladder (Genei) on 1% agarose gel containing ethidium bromide. Bands were visualized and photographed using an UV transilluminator and a gel documentation apparatus (Alpha Innotech Corp, CA, USA).

For cloning of PCR products, the expected size bands were excised from the gel and eluted using Perfectprep Gel Cleanup kit (Eppendorf, Germany). These eluted bands were cloned in T/A cloning vector (Genei) and *Escherichia coli* DH-5α were transformed. Recombinant clones were identified by restriction digestion. Three clones each of CMV and PYMoV obtained by independent cloning events were sequenced at Avestha Gengraine Pvt Ltd, Bangalore.

Initially, plants were tested for the presence of CMV and PYMoV separately by RT–PCR and PCR respectively, using specific primers. Plants showing mixed infection with CMV and PYMoV were selected for mRT–PCR studies. Total nucleic acids extracted from mixed infected plants when subjected to mRT–PCR, showed the presence of two PCR products of expected size (650 bp for CMV and 450 bp for PYMoV). No such bands were observed in healthy plants (Figure 1). Further identity of amplicons was confirmed by their sequencing and analysis with published sequence data available in GenBank. Sequence analyses showed that the amplicon for CMV was 657 bp and matched exactly with CMV (AY545924). Similarly, the amplicon for PYMoV was 450 bp and matched with the sequence of PYMoV (DQ836226).

In order to determine the optimum template volume required for effective amplification, mRT–PCR was set up using a range of template volumes (10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1.0 and 5.0 µl). Results showed that detection level of both viruses varied depending on the template volume. PYMoV was detectable at all template volumes used, though better detection was observed in template volumes ranging from 10^{-2} to 5.0 µl (Figure 2). In contrast, CMV could be detected only at higher template volumes ranging from 10^{-2} to 5.0 µl. For simultaneous detection of both viruses, optimum template volume was found to be in the range 10^{-1}–1 µl in a 50 µl reaction, where both bands were strong and clearly visible (Figure 2).

The optimized detection procedure was used for screening viruses in black pepper samples collected from different geographical regions of Kerala and Karnataka. Of the total 49 samples tested, 34 were positive for virus infection. Among these, three samples were positive for combined

Table 1. Sequences of oligonucleotide primers designed for amplification of CMV and PYMoV infecting black pepper using multiplex RT–PCR

Primer name	Sense	Sequence (5′···3′)	Region amplified	Amplicon size (bp)	Accession no.
CMV-F	Forward	ATGGACAAATCTGAATCAAC	Coat protein gene of CMV	650	AY545924
CMV-R	Reverse	TCAAACTGGGAGCACCC			
PYMoV-F	Forward	TAACAGGACTAGGGATCG	Portion of ORF I of PYMoV	450	DQ836226
PYMoV-R	Reverse	CAGCTGGTCTTGATAATAG			

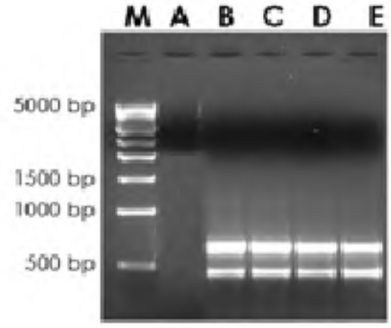

Figure 1. Agarose gel electrophoresis profile of amplicons obtained by single-tube mRT–PCR using total nucleic acid extracted from black pepper. Lane M, 500 bp DNA ladder; lane A, Healthy black pepper, and lanes B–E, Infected plants.

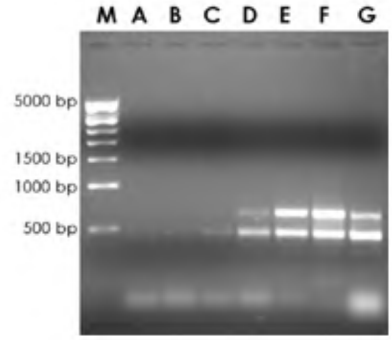

Figure 2. Determination of optimum template volume required for mRT–PCR in detecting CMV and PYMoV from black pepper. Lane M: 500 bp DNA ladder, and lanes A–G, Template volumes of 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1 and 5 µl respectively.

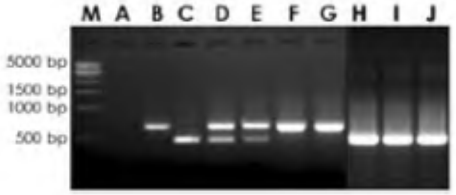

Figure 3. Detection of CMV and PYMoV from field samples of black pepper using mRT–PCR. Lane M, 500 bp DNA ladder; lane A, Healthy plant; lane B, Plant infected with CMV alone; lane C, Plant infected with PYMoV alone, and lanes D–J, Test plants.

infection, 24 were positive for PYMoV alone and seven were positive for CMV alone. No virus amplification was observed in the rest of the samples. A representative gel picture showing detection of viruses in field samples is presented in Figure 3.

Stunt disease of black pepper reduces the yield and vigour and occupied the third place among serious diseases of black pepper[3]. Due to sensitivity and efficacy, PCR-based diagnostics are preferred for determining the healthy planting material. We have standardized a protocol for the isolation of total RNA from black pepper[21]. This protocol was further modified to isolate DNA from the interphase. To obtain total nucleic acids in a single tube, both nucleic acids (DNA and RNA) were combined in the isopropanol step to co-precipitate in a single tube. Detection of viruses at low template volumes suggests the effective extraction of amplifiable amount of total nucleic acids from the tissue. The use of optimum template volume was found to be crucial for successful amplification of both viruses. PCR-based detection of more than one RNA/DNA virus was reported from different crops[14–19]. Though simultaneous detection of an RNA and DNA virus was reported from rice, templates were generated only using commercial kit[19]. The present study reports the total nucleic acid isolation procedure that provides amplifiable templates for mRT–PCR-based detection of two distinct viruses. Thus we report the simultaneous isolation and detection of RNA and DNA viruses infecting black pepper.

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Bryophyte diversity along a gradient of human disturbance in the southern Western Ghats

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Evergreen forests, degraded evergreen forests, clove and rubber plantations were sampled for bryophyte species diversity in the Southern Western Ghats. Evergreen forests have the highest diversity followed by degraded evergreen forests, clove and rubber plantations. Besides, evergreen forests have the highest number of exclusive species whereas degraded evergreen forests, clove and rubber plantations have more generalized and light-tolerant species such as *Fissidens ceylonensis*, *Hyophila comosa*, *H. involuta* and *Frullania acutiloba*. Monoculture plantations and degraded evergreen forests as a result of human interference enable the invasion of such generalized and light-tolerant species which would sooner or later replace species with restricted distribution in evergreen forests if human interference continues unabated.

Keywords: Bryophytes, diversity, human disturbance, natural forests, plantations, Western Ghats.

BRYOPHYTES are nonvascular plants generally reproducing by spores. They are considered to be pioneers that colonize terrestrial habitats from an aquatic environment. They are the simplest and the most primitive of the land plants as they do not have a well developed conductive tissue system. Though basically terrestrial, there are a few aquatic forms such as *Riccia fluitans*, *Ricciocarpus natans* and *Riella* spp. *Cryptothallus* and *Buxbaumia* are saprophytic genera of liverworts and mosses respectively. Bryophytes are more common in humid areas and during rainy seasons, but usually show a preference for microclimatic niches such as crevices of rocks and trees and the vicinity of small shady springs. However, they can grow on a wide range of substrata. They may be found on old discarded/abandoned leather goods, rubber tyres, wooden articles, tiled and asbestos roofs and mortar of stone and mud walls. They also grow as epiphytes on barks of trees (corticolous), leaves (folicolous), rocks (rupicolous), stones and pebbles (saxicolous), fallen logs (lignicolous), river banks and roadside cuts (terricolous). Since water is inevitable for completing their life cycle, they are known as the 'amphibians' of the plant kingdom. However, many are drought-tolerant and are secondary colonizers on barren rocks in a xerosere after lichens. With a remarkable capacity to absorb water they turn fresh in no time and hence are also known as 'resurrection plants'.

India, one of the 12 megabiodiversity countries of the world, possesses a large area and a variety of phytoclimatic conditions which contribute to the great diversity of the flora. Pandé[1] divided these zones into seven bryogeographical regions, namely the Western and Eastern Himalayas, Punjab and West Rajasthan, Gangetic Plains, Central India, Deccan Plateau, and the Western and Eastern Ghats. The flora of the Western Ghats has been studied with emphasis on flowering plants and even pteridophytes. However, knowledge on the taxonomy, ecology and distribution of bryophytes is still far from adequate[2]. Moreover, questions like whether monoculture plantations lead to reduction of species diversity and ultimately local extinction of species remain unanswered. The present study is an effort to answer such questions and to highlight the need, for conservation of natural ecosystems.

Kanyakumari District, Tamil Nadu is situated at the southernmost tip of Peninsular India. It lies between 8°5′ and 8°30′N lat.; 77°10′ and 77°36′E long. The geographical position of the district is such that it receives both southwest and northeast monsoons. Normally during December

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