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Occurrence of *Banana bract mosaic virus* on cardamom

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Abstract During a survey for mosaic disease caused by *Cardamom mosaic virus* (CdMV) in cardamom plantations, a new kind of viral disease showing chlorotic streak on veins was observed in some plantations. Based on the type of symptom, the disease was named 'chlorotic streak'. In a survey of 77 cardamom plantations at 49 locations in the states of Kerala, Karnataka and Tamil Nadu in India, incidence of the disease ranged from zero to 15%. The plants infected with chlorotic streak tested negative in reverse transcription polymerase chain reaction (RT-PCR) for CdMV. Leaf dip electron microscopy of these samples showed the presence of flexuous virions resembling *Potyvirus*. Total RNA extracted from these plants when subjected to RT-PCR using primers targeted to conserved region in *Potyvirus* resulted in an amplicon size of ~700 bp. Sequencing and BLAST analysis of this amplicon showed *Banana bract mosaic virus* (BBrMV) as the closest virus. Using primers specific for BBrMV, the coat protein gene from five different geographical isolates of the virus was cloned and sequenced. Sequence analysis of the coat protein gene showed an identity of >94% with BBrMV isolates while identity with other distinct potyvirus species was <60%, indicating that the causal virus is a strain of BBrMV. Multiple sequence alignment and phylogenetic analyses showed high sequence conservation and close clustering of cardamom isolates along with a few banana isolates from India. This is the first report of the occurrence of BBrMV in cardamom. A reliable RT-PCR-based method was also developed for detection of the virus in cardamom plants.

Keywords BBrMV · *Cardamom mosaic virus* · Chlorotic streak · Coat protein · Detection · *Elettaria cardamomum* · India · RT-PCR

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Introduction

Banana bract mosaic disease caused by *Banana bract mosaic virus* (BBrMV) was first observed in banana plants in the Philippines during 1979 and is currently reported from Costa Rica, India, Sri Lanka, Western Samoa, Vietnam and Thailand (Bateson and Dale 1995; Espino *et al*. 1990; Rodoni *et al*. 1999; Thomas *et al*. 1997). Based on the presence of uncharacterized potyvirus-like particles, Rodoni *et al*. (1999) suspected its occurrence in several African countries including Uganda, Ghana, Zanzibar and South Africa.

The disease is characterized by mosaic symptoms on flower bracts, irregularly scattered spindle-shaped streaks along the petiole and mottled discoloration on the pseudostem. No other hosts of the virus than banana plants have been reported so far and the virus is not mechanically transmitted. It is transmitted primarily through infected suckers and secondary spread is by aphids in a non-persistent manner (Magnaye and Espino 1990; Rodoni *et al*. 1999).

Cardamom (*Elettaria cardamomum* Maton) is a large perennial, herbaceous rhizomatous monocot, belonging to the family *Zingiberaceae.*The cardamom of commerce is the dried fruit (capsules) of the cardamom plant. Cardamom is grown in India, Sri Lanka, Papua New Guinea, Tanzania and Guatemala. In India, cardamom is grown in the hilly regions of Kerala, Karnataka and Tamil Nadu mostly as a monocrop under the shade of forest trees (Ravindran 2002). Mosaic disease (locally known as *katte*/*marble*) caused by *Cardamom mosaic virus* (CdMV) (genus: *Macluravirus*) is an important viral disease affecting cardamom in India, Guatemala and Sri Lanka (Dimitman *et al*. 1984; Gonsalves *et al*. 1986; Mayne 1951; Venugopal 2002). During our survey for mosaic disease in cardamom, a novel kind of viral symptom was observed in certain plantations. The disease was characterized by continuous or discontinuous chlorotic streak along the veins and midrib and hence named as 'chlorotic streak'. These plants tested negative for CdMV in RT-PCR. In this article, we report the incidence of chlorotic streak disease, its identification, partial molecular characterization and development of RT-PCR-based detection for the virus in cardamom plants.

Materials and methods

Distribution and incidence of disease A total of 77 cardamom plantations found in 49 geographical locations of the Karnataka, Kerala and Tamil Nadu states of India were surveyed, to determine the distribution and incidence of chlorotic streak disease of cardamom. The survey was conducted according to the method used by Govindaraju *et al.* (1994). In each selected plantation, disease incidence was recorded based on the observations on a cluster of 100 plants. The number of clusters in each plantation was fixed on the basis of the effective area under cardamom cultivation: 2 ha=5 clusters; 2.1 to 5 ha=10 clusters; 5.1 to 10 ha=15 clusters; and more than 10 ha=20 clusters. The disease incidence in each plantation was calculated after counts of all clusters were pooled.

Identification and characterization of the virus

Virus isolates Disease-affected cardamom samples collected from Sirsi (Uttara Kannada), Mudigere (Chikmagalur), Somwarpet (Kodagu) of Karnataka state; and Meppadi (Wynad) and Vellathooval (Idukki) of Kerala state were used for the identification and characterization of the virus. In addition, many infected and apparently healthy cardamom samples collected from different areas of Karnataka and Kerala, India, were used in the detection studies through RT-PCR. Cardamom plants raised from seeds under insect-proof conditions were used as a source of a healthy (negative) control.

Electron microscopy Electron microscopy of leaf dip preparations negatively stained with 2% uranyl acetate (pH 4.5) was carried out using a JEOL 100 CFII transmission electron microscope at the Unit of Plant Virology, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi.

Primer designing Potyvirus genus-specific primers for the conserved 'WCIEN' and Poly (A) region were synthesized as per Pappu *et al*. (1993). To amplify the coat protein (CP) gene of BBrMV, forward and reverse primers were designed to the conserved region identified based on multiple sequence alignment of CP gene and 3′ untranslated region (UTR) of all available BBrMV sequences from the GenBank database. The forward primer (5′ TCTGGAACG GAGTCAAC 3′) represented the first 17 bases of the CP gene and reverse primer (5′ GCACACATAAT TATAGGGAG 3′) was derived from the 3′ UTR.

Total RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) Total RNA was isolated from 100 mg of leaf tissue following the method described by Siju *et al.* (2007). RT-PCR was performed in a single tube without any buffer changes in between. RT-PCR reaction contained 20 pmol of each of the forward and reverse primers, 10 U ribonuclease inhibitor (Fermentas, Glen Burnie, MD, USA), 20 U of M-MuLV reverse transcriptase (Fer-

mentas), 1.5 U *Taq* DNA polymerase (Fermentas), 1x PCR buffer (Genei, Bangalore, India), 10 mM dithiothreitol (Genei) and 5 μM each of the dNTPs (Genei). A RT-PCR mixture (30 μl) containing the above components was added to tubes containing template RNA (20 μl) resulting in a final reaction volume of 50 μl. The RT-PCR profile using *Potyvirus* genus-specific primers (Pappu *et al.* 1993) consisted of one cycle at 42°C for 45 min for cDNA synthesis followed by 35 cycles reaction profile involving 30 s of denaturation at 94°C, 2 min of annealing at 42°C and 1 min of extension at 72°C and a single cycle of final extension at 72°C for 10 min. The RT-PCR profile for the BBrMV-specific primers was the same as above except that annealing was carried out at 52°C for 1 min. The reaction products were analyzed on 0.8% agarose gel along with 1 kb DNA ladder. The DNA bands were visualized and photographed using a UV transilluminator and a gel documentation apparatus (Cell Biosciences, Santa Clara, CA, USA).

Cloning, sequencing and sequence analyses The PCR product obtained was eluted from the gel using GenElute Gel Elution kit (Sigma-Aldrich, Bangalore, India), cloned into pTZ57R/T cloning vector (Fermentas) and transformed into competent *E. coli* strain DH5α using InsTAclone PCR cloning kit (Fermentas) following the manufacturer's instructions. Recombinant clones were identified by PCR as well as restriction endonuclease digestion and selected clones were sequenced from both the directions at the automated DNA sequencing facility available at Chromous Biotech, Bangalore, India. Sequence data were compiled using Seqaid Version 3.6 (Rhoads and Roufa 1985). Multiple sequence alignments were made using ClustalX (1.81) and trees were generated using TreeView software. Percent identities were determined using the Bioedit program version 5.0.9. The CP nucleotide and amino acid sequences of other BBrMV isolates used for comparison were obtained from GenBank (Benson *et al.* 1999). The BLAST programme (Altschul *et al.* 1997) was used to identify related sequences available from the GenBank database.

Development of RT-PCR-based detection for virus Total RNA from cardamom leaves was isolated by using the method described earlier. Based on multiple alignments of CP gene and 3′ UTR sequences of different isolates of BBrMV infecting cardamom obtained in the present study, conserved regions were identified. Forward primer (5′ GCACATTTGGA CAACCCA 3′) derived from 105–123 bases from the highly conserved core region of CP gene and reverse primer (5′ GCACACATAATTATAGGGAG 3′) derived from 3′ UTR were used. RT-PCR components and profile were the same as described earlier except that the annealing step was carried out at 54°C for 1 min. For initial standardization of the RT-PCR method, known infected samples from five different regions and healthy cardamom samples were used. Positive reactions were identified by the presence of 850 bp product specific for BBrMV. Identity of the amplicons (from five different geographical regions) was confirmed by directly sequencing the gel-purified PCR products. Absence of the expected amplicon indicated a negative reaction and thus, absence of the virus. In order to validate the RT-PCR method, 40 cardamom samples collected from different regions of Karnataka and Kerala were used. To examine the reliability of the method, especially to detect the virus in symptomless plants, samples taken from apparently healthy suckers from BBrMV-infected clumps and apparently healthy plants around the infected plants were used.

Results

Distribution and incidence of the disease Of 77 plantations in 49 locations surveyed, incidence of the disease ranged from zero to 15%. In Kerala, of 44 plantations in the 30 locations surveyed, the disease incidence ranged from zero to 15% (Table 1). The highest disease incidence of 15% was recorded at Vythiri Taluk of Wynad District, whereas no disease was recorded in Palakkad District. In Karnataka, disease incidence ranged from zero to 5% with the highest incidence (5%) in Sirsi, Siddapur and Yellappur Taluks of Uttara Kannada District, and no disease recorded in Madikeri and Virajpet Taluks of Kodagu District, or Sakleshpur Taluk of Hassan District. Similarly, in Tamil Nadu chlorotic streak was not found in any of the plantations surveyed.

Only in Uttara Kannada district of Karnataka state was cardamom grown as a mixed crop with arecanut and banana. In this region, incidence of BBrMV in

Table 1 Distribution and incidence of chlorotic streak disease caused by *Banana bract mosaic virus* in major cardamom-growing regions of India

State/ District	Administrative division	No. of locations surveyed	No. of fields surveyed	Disease incidence	
				Range (%)	Mean
Karnataka					
Uttara Kannada	Sirsi	3	4	1–5	2.75
	Siddapur	1	3	0–5	1.6
	Yellapur	3	3	1–5	2.0
Kodagu	Madikeri	3	7	0	0
	Somwarpet	2	4	0–4	1.0
	Virajpet	1	2	0	0
Hassan	Sakleshpur	3	3	0	0
Kerala					
Idukki	Udumbanchola	17	37	0–12	2.13
	Peermade	4	4	0–6	1.75
	Devikulam	5	7	2–12	2.4
Wynad	Vythiri	3	4	5–15	11.0
Palakkad	Chittoor	2	4	0	0
Tamil Nadu					
Coimbatore	Valparai	1	4	0	0
Dindigul	Kodaikkanal	1	2	0	0
Salem	Yercaud	1	1	0	0

banana ranged from 2% to 35%. In the rest of the surveyed areas cardamom was grown as a monocrop although in some regions a banana plantation was seen adjoining cardamom plantations, and in those regions the incidence of BBrMV in cardamom was particularly high. A low incidence of BBrMV was also seen in cardamom grown far away from banana, which could have been due to the movement of suckers from an infected area.

Symptomatology The disease was characterized by continuous or discontinuous spindle-shaped yellow or light green intravenous streaks along the veins and midrib (Fig. 1a). These streaks later coalesce together and impart a yellow or light green color to veins (Fig. 1b). Discontinuous spindle-shaped mottling on the pseudostem and petioles was also noticed (Fig. 1c). In severe cases, tillering in an infected plant was suppressed (Fig. 1d). The distinguishing feature of the present disease was formation of intravenous chlorotic streaks; hence the disease was named 'chlorotic streak'. Symptoms on infected banana included spindle-shaped lesions on leaf lamina and petiole and dark reddish-brown streak or mosaic pattern on the pseudo sheath and bracts of the inflorescence.

Identification and characterization of the causal virus Leaf dip electron microscopy of infected samples showed the presence of flexuous virions resembling *Potyvirus*. However, samples had a negative reaction for CdMV in RT-PCR. In view of this, total RNA extracted from these plants was subjected to RT-PCR using primers for the conserved region in *Potyvirus* (WCIEN) and Poly (A) which resulted in an amplicon size of ~700 bp (not shown).

Sequencing and BLAST analysis of this fragment showed BBrMV as the closest relative. Hence, primers specific for BBrMV to amplify the CP gene were designed through multiple alignment of all available BBrMV sequences from the GenBank database. RT-PCR performed with BBrMV specific primers resulted in an amplicon of ~950 bp in all five samples collected from different geographical regions whereas no product was observed in the healthy control (Fig. 2). All these samples tested negative when CdMV-specific primers were used in RT-PCR. The

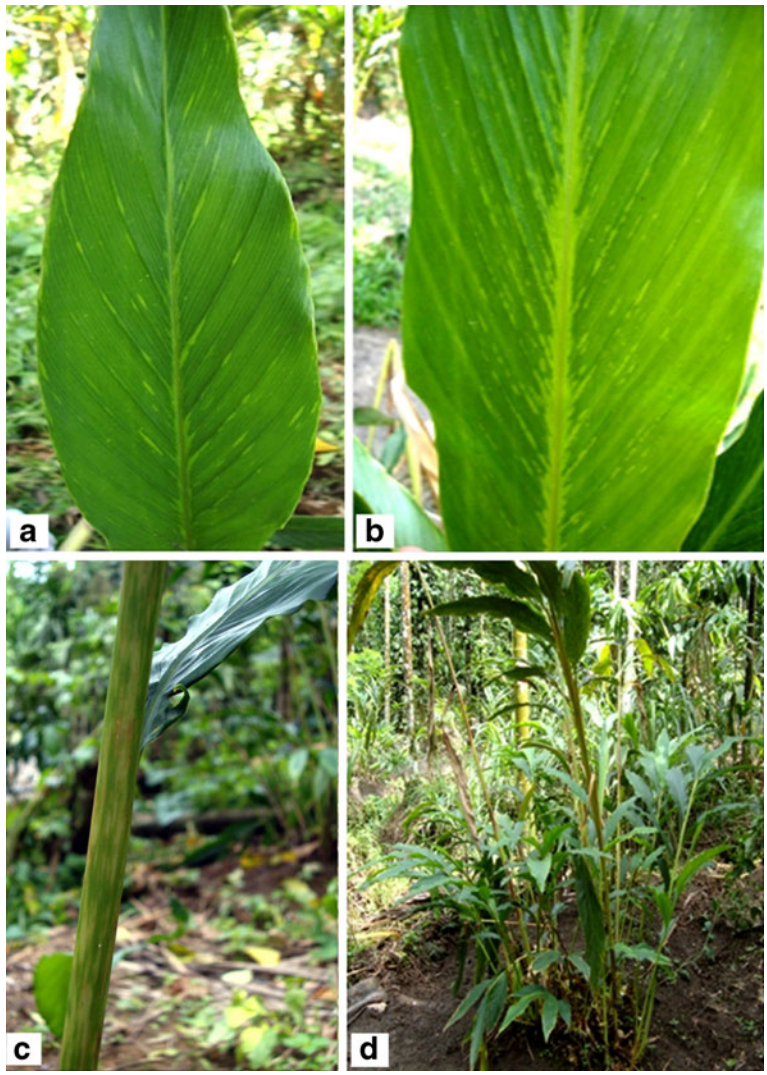

Fig. 1 Symptomatology of chlorotic streak disease of cardamom (*Elettaria cardamomum*). (**a**) Spindle-shaped yellow or light green intravenous streaks along the midrib and veins; (**b**) advanced stage showing light green or yellow veins; (**c**) spindle-shaped mottling along the pseudostem; (**d**) reduced tillering in an affected clump

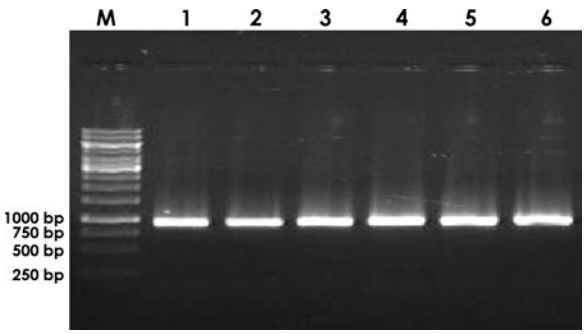

Fig. 2 Agarose gel showing RT-PCR amplification of cardamom (*Elettaria cardamomum*) samples using *Banana bract mosaic virus*-specific primers. Lane M: Marker (1 Kb ladder); Lanes 1–5: chlorotic streak-infected cardamom samples from five different geographical areas; Lane 6: positive control (BBrMV-infected banana sample)

amplified product from all five isolates was cloned and sequenced. The sequenced region contained 945 nucleotides in all five isolates in which first 900 bp represented CP region potentially coding for 300 amino acid residues, while the remaining 45 bases represented 3' UTR. The sequences were deposited in GenBank and their accession numbers are provided in Table 2. The CP gene sequences of isolates were compared among themselves and with corresponding CP gene sequences of all available BBrMV isolates (Table 2) and a few other potyviruses. Nucleotide sequence identity among cardamom isolates sequenced in the present study varied from 97–99% and 96–99% at nucleotide and amino acid levels, respectively. Sequence analysis with all available BBrMV isolates infecting banana and cardamom

Table 2 Host, geographical origins and GenBank accession numbers of the *Banana bract mosaic virus* isolates used in this study

Designation	Country (Region)	Host	Accession No.
Card-1	India (Madikeri, Karnataka)	Cardamom	HQ709165
Card-2	India (Mudigere, Karnataka)	Cardamom	HQ709166
Card-3	India (Sirsi, Karnataka)	Cardamom	HQ709164
Card-4	India (Sirsi, Karnataka)	Cardamom	AY776327
Card-5	India (Idukki, Kerala)	Cardamom	HQ709163
Card-6	India (Wynad, Kerala)	Cardamom	HQ709162
Ban-1	India (Coimbatore, Tamil Nadu)	Banana	AF071582
Ban-2	India (Coimbatore, Tamil Nadu)	Banana	AF071583
Ban-3	India (Coimbatore, Tamil Nadu)	Banana	AY494979
Ban-4	India (Trichy, Tamil Nadu)	Banana	AF071584
Ban-5	India (Trichy, Tamil Nadu)	Banana	EU009210
Ban-6	India (West Godavari, Andhra Pradesh)	Banana	AY953427
Ban-7	India (Karnataka))	Banana	EF654655
Ban-8	India	Banana	EU699770
Ban-9	Philippines (Los Baños)	Banana	AF071590
Ban-10	Philippines (Los Baños)	Banana	AF071585
Ban-11	Philippines (Davao)	Banana	AF071586
Ban-12	Philippines	Banana	DQ851496
Ban-13	Philippines	Banana	EU414267
Ban-14	Thailand	Banana	AF071589
Ban-15	Vietnam (Hau)	Banana	AF071588
Ban-16	Western Samoa (Nu'u)	Banana	AF071587

showed an identity ranging from 94–100% and 95–100% at nucleotide and amino acid sequence, respectively (not shown).

Identity within banana isolates of BBrMV ranged from 94–100% and 96–100% at nucleotide and amino acid levels, respectively. Most of the differences were seen in the N-terminal region of the CP. Six BBrMV banana isolates from India showed high identity towards BBrMV cardamom isolates (97–99%) while BBrMV banana isolates from Philippines, Thailand, Vietnam, Western Samoa along with two Indian isolates showed identity of 94–96.8% with cardamom isolates. BLAST analysis showed *Sugarcane mosaic virus* (SCMV) as the closest species to the present virus. Identity in the CP gene sequence of SCMV with present virus isolates was 60% at nucleotide and 55% at amino acid levels. Identity in the CP gene with CdMV infecting cardamom was only 23% at nucleotide and amino acid levels, clearly indicating their difference at the genetic level. High CP sequence identity of the present isolates (>94%) with BBrMV indicates that the causal virus associated with chlorotic streak disease is a strain of BBrMV.

Multiple sequence alignment of CP gene deduced amino acid revealed a slightly variable N-terminal region that had maximum sequence heterogeneity while the core and C-terminus was found to be highly conserved. Differences of two to six amino acids were observed among cardamom isolates. Analysis of the sequence revealed several motifs commonly found in potyviral coat proteins including a DAG amino acid triplet, a WCIEN box in the core region and an RQ, AFDF and QMKAA. A phylogenetic tree was constructed using nucleotide sequences of the entire CP coding region of 22 BBrMV isolates (Fig. 3). Phylogenetic analyses of the BBrMV isolates revealed two major clusters, one with all cardamom along with six banana isolates from India while the other cluster consisted of banana isolates from the Philippines, Thailand, Vietnam, Western Samoa and two from India.

Development of RT-PCR based detection for virus In the initial standardization, expected size of ~850 bp was observed in all the five infected plants while no such band was observed in the healthy plants. Specificity of

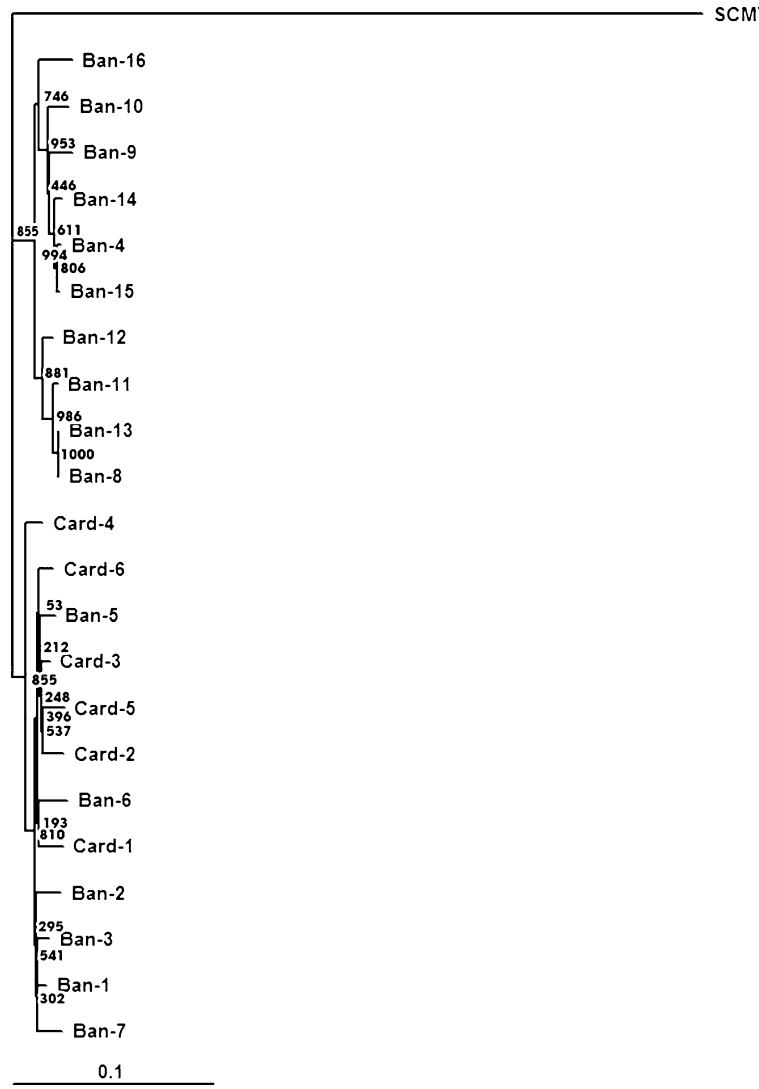

Fig. 3 Phylogram drawn by Neighborhood Joining method in Clustal X (1.81), illustrating phylogenetic relationships based on the multiple alignments of nucleotide sequences of 22 distinct isolates of *Banana bract mosaic virus* infecting banana and cardamom. *Sugarcane mosaic virus* (SCMV) was used as outgroup. The bootstrap values are shown at the individual nodes

Table 3 Detection *via* RT-PCR of *Banana bract mosaic virus* from field samples of cardamom (*Elettaria cardamomum*) collected from different regions of India

State	District	No. of samples tested			No. of positive samples in RT-PCR		
		Symptomatic	Symptomless tillers (from infected clump)	Symptomless plants (around infected clump)	Symptomatic	Symptomless tillers (from infected clump)	Symptomless plants (around infected clump)
Karnataka	Kodagu	2	0	1	2	0	0
	Hassan	3	0	1	3	0	0
	Uttara Kannada	8	2	2	8	2	0
Kerala	Wynad	3	3	3	3	3	1
	Idukki	6	3	3	6	3	0
Total		22	8	10	22	8	1

the primers in detecting BBrMV alone was confirmed by including known CdMV-infected cardamom plants in the tests which showed no amplification. Identity of the amplicon from all the five isolates was also confirmed by directly sequencing the gel purified PCR product which matched with the respective sequences obtained in the present studies (not shown). Results on the validation of the RT-PCR method using 40 field samples showed successful detection of BBrMV in 31 samples (Table 3). All symptomatic samples (22) collected from different geographical regions tested positive for the virus. Of the eight asymptomatic tillers collected from different infected clumps of cardamom, all samples tested positive indicating BBrMV infection in these tillers. Of the ten apparently healthy plants, collected from adjacent infected plants, one tested positive for BBrMV infection. RT-PCR was also able to detect BBrMV in infected banana plants around cardamom plantations.

Discussion

The present study reports association of BBrMV with cardamom plants affected with chlorotic streak disease. The symptoms induced by BBrMV on cardamom were distinct and easily distinguishable from the symptoms due to other viral diseases reported to affect cardamom (Venugopal 2002). The disease was characterized by the appearance of chlorotic streaks along the veins and hence we propose the name 'chlorotic streak' for the disease. Studies on the occurrence and distribution of the disease showed an incidence ranging from 0-15% in different cardamom-growing regions. The incidence of the disease was higher in plantations where either banana was grown nearby or banana had been the previous crop. BBrMV is transmitted primarily through suckers and secondary spread is by aphids (Magnaye and Espino 1990; Rodoni *et al.* 1999). The aphid *Pentalonia nigronervosa,* known to exist and breed both in banana and cardamom plantations (Venugopal 2002), might be responsible for the horizontal transmission of the virus from banana to cardamom or *vice versa*. The causal virus was identified by cloning and sequencing of the CP gene of the virus. The CP gene from different isolates of the virus showed >94% identity with BBrMV, clearly indicating that the causal virus is a strain of BBrMV. So far BBrMV is reported to infect only banana (Diekmann and Putter 1996). This is the first report of the occurrence of BBrMV in cardamom. Comparison of the CP gene sequences of BBrMV isolates infecting cardamom showed a high level of sequence conservation (96–99%) among the isolates. Phylogenetic analysis revealed clustering of cardamom isolates with six banana isolates from India while banana isolates from other countries along with two Indian isolates formed a separate cluster. Sequence conservation of 94.4–99.7% was reported among BBrMV isolates infecting banana from different banana-growing countries (Rodoni *et al.* 1999). In the present study, using CP gene sequence of all available BBrMV isolates infecting banana and cardamom, an identity ranging from 94–100% among isolates was found.

Detection of virus is an important component in effective management, especially if the virus is transmitted primarily through seeds or vegetatively. As cardamom is mainly vegetatively propagated through tillers, it is important to identify virus-free plants, to be used as mother plants for further propagation. As symptoms alone cannot be reliable criteria due to a variable incubation period of the virus depending on the cultivar and season, sensitive techniques such as RT-PCR may be needed to detect BBrMV infection, especially in tillers as observed in the present study. The protocol standardized for RNA isolation and RT-PCR can be used for indexing cardamom plants to identify BBrMV-free plants and also for identifying lines/cultivars resistant against BBrMV.

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