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Research Article **Plant Virus**

Detection and partial nucleotide sequence analysis of *Piper yellow mottle virus* infecting black pepper in India

P.S. HAREESH and A.I. BHAT
Division of Crop Protection, Indian Institute of Spices Research, Marikunnu. P.O., Calicut-673012

ABSTRACT

A portion of viral genome corresponding to open reading frame (ORF) I and III of the *Badnavirus* associated with stunted disease of black pepper (*Piper nigram* L.) in India was cloned and sequenced. The sequenced region of ORF I contained 694 to 710 bases while that of ORF III contained 597 to 600 bases in different isolates. Sequence analysis and phylogram confirmed that the identity of the virus as a strain of *Piper yellow mottle virus* (PYMoV). Further, high level of sequence conservation (=95%) was noticed in the sequenced region of both ORF I and ORF III in three isolates collected from different regions. A reliable protocol was also developed for the isolation of DNA from black pepper and PCR for detection of the virus to identify the virus-free plants of black pepper.

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Corresponding author: A.I. Bhat (aib65@yahoo.co.in)
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INTRODUCTION

The genus *Badnavirus*, that belongs to the family *Caulimoviridae* (Pringle,1998), have bacilliform shaped virions measuring ~30 x 130 -150 nm in size and a circular covalently closed dsDNA genome (Lockhart 1995). Majority of the badnaviruses have three open reading frames (ORFs). The ORF III codes for a polyprotien that is cleaved into functional subunits such as movement protein, reverse transcriptase, coat protein and RNase H by the aspartic protease while functions of ORF I and II are not known (Hohn and Futterer, 1997). However the products of ORF I in *Commelina yellow mottle virus* (ComYMV) was demonstrated to be virion associated (Cheng *et al.,* 1996) and that ORF II of *Cacao swollen shoot virus* (CSSV) was reported to have nucleic acid binding property (Briddon *et al*., 1999).

Black pepper popularly known as 'black gold' is one of the most ancient spice crops cultivated for its berries (Ravindran, 2000). In India, the core black pepper cultivating regions are confined to the southern states of Kerala and Karnataka. Stunted disease caused by viruses is one of the major production constraints of black pepper in India, Brazil and many south East Asian countries (Pailey *et al*., 1981; Lockhart *et al*., 1997; Sarma *et al*., 2001; Bhat *et al*., 2003). The disease is characterized by leaf distortion, mosaic, mottling, shortened internodes, and poor filling of spikes leading to reduced yield. Though the disease was referred by different names in different black pepper growing countries, at the 1991 international workshop on black pepper diseases held at Lampung, Indonesia, it was decided to use "Stunted disease" as a uniform terminology to include all viral diseases (Wahid *et al*., 1992). Lockhart *et al.* (1997) reported a new mealy bug transmitted badnavirus, namely *Piper yellow mottle virus* (PYMoV) as cause of the disease in Malaysia, Thailand, The Philippines and Sri Lanka. PYMoV had non-enveloped bacilliform virions measuring ~ 30 x 125 nm containing a double stranded DNA genome. In addition to PYMoV association of *Cucumber mosaic virus* (CMV) was also reported with the disease on black pepper from Sri Lanka (de Silva *et al*., 2002), Brazil, Indonesia (Suastika *et al*., 2005). In India too, involvement of CMV belonging to subgroup IB (Sarma *et al*., 2001; Bhat *et al*., 2005) and a mealybug transmitted *Badnavirus* was shown to be associated with the disease (Bhat *et al*., 2003). However exact taxonomic identity of the *Badnavirus* infecting black pepper in India remained unaddressed. In this article we report the identification of the virus based on cloning and sequencing of a portion of ORF I and ORF III. Sequence variability existing in these genomic regions among three different geographical isolates of the virus was also studied. Further as black pepper is clonally

propagated by vegetative means, a reliable protocol for DNA isolation and PCR based detection was developed for identification of virus-free planting material.

MATERIALS AND METHODS

Virus isolates

Stunted disease affected black pepper samples collected from Calicut and Idukki, district of Kerala and Coorg district of Karnataka, India was used in the study. All these isolates were maintained through vegetative propagation under insect proof glass house conditions. Besides, many nursery and field black pepper plants collected from different areas of Karnataka and Kerala, India were used in the detection studies through PCR. Black pepper plants raised from seeds under insect proof condition was used as source of healthy (negative) control while symptomatic and PCR positive plants were used as positive control.

Primer designing

To amplify the genome of *Badnavirus* infecting black pepper, a total of 12 oligonucleotide primers were synthesized either based on the sequence of primers available in the literature (Lockhart *et al*., 1997; de Silva *et al*., 2002) or the conserved sequence region identified by multiple alignments generated by Clustal X, using complete genome sequences of different badnavirus isolates from GenBank (Benson *et al*., 1999). The list of synthesized primers is given in Table 1.

Amplification, cloning and sequencing

Total DNA from stunted disease affected black pepper samples was extracted using DNeasy Plant mini kit (Qiagen). The PCR reaction (50µl) contained 50 ng each of the primers, 1.5 U *Taq* DNA polymerase (Genei, Bangalore, India), 1x PCR buffer (Taq Buffer B, Genei, Bangalore, India), 2.5mM $MgCl_2$ (Genei, Bangalore, India) and 200µM dNTPs (Finnzymes OY, Finland) and 31.5µl of sterile water. PCR mix (45µl) containing the above components was added to the tubes containing the template DNA (5µl) resulting in a final reaction volume of 50µl. The PCR profile using the primers SCBVR1 and Badna 1R involved initial denaturation at 94 ^{0}C for 5 min followed by 35 cycles with denaturation at 94 ^{0}C for 30s, annealing at 56 ^{0}C for 1 min, synthesis at 72 ^{0}C for 1 min and a final extension of 10 min at 72 ^{0}C. The PCR profile for the primers Badna 3F2 and Badna 3R1 were same as above except that annealing was carried out at 58 ^{0}C. The amplified products were loaded on to a 1% agarose gel containing ethidium bromide (Sambrook and Russel, 2001) in 1x TAE buffer and run at 60V (Hoefer

Table 1. List of primers used in PCR. Universal codes for degenerate bases R (A,G), Y (C,T), M (A,C), K (G,T), S (G,C), W (A,T), H (A,T,C), B (G,T,C), V (G,A,C), D (GAT), N (A,C,G,T)

Primer name	Primer sequence (5'……3')	Region	Nucleotide position with reference to SCBV-Mor
Badna 2	TAYATHGAYGAYATHYT	Badna conserved region in RT, Forward primer (Lockhart *et al.,* 1997)	5784-5800
Mys3	CCCCATRCANCCRTCNGTYTC	Badna conserved region in RNase H domain Reverse primer (Lockhart *et al.,* 1997)	2094-2114
Badna 3	ATHATHATHGARACYGAY	Badna conserved region in RNAse H domain Forward primer (Lockhart *et al.,* 1997)	6222-6239
Badna-T	MYMWNGCTCTGATACCA	Badna conserved region in tRNA binding site Reverse primer (Lockhart *et al.,* 1997)	770-786
SCBV-R1	CTCCTTCATCTCCTCAAGAAGCCT	PYMoV ORF I, Forward primer (de Silva *et al.,* 2002)	562-585
Badna 1R	CCAAAGCTCTGATAGCAGAC	PYMoV ORF I, Reverse primer (de Silva *et al.,* 2002)	1771-1790
Badna 3FI	AAG TTY GAYYTRAA RWSB GG	Badna ORF III, Forward primer (Based on multiple sequence alignment) (This study)	5594-5614
Badna 3F2	CTNTAGAATGGYTWGTDATGCC	-do-	5679-5702
Badna 3F3	TRTTYCARMGRAAARATGGA	-do-	5728-5736
Badna 3R1	CCAYTTRCAKAYKSCHCCCC	Badna ORF III, Reverse primer (Based on multiple sequence alignment) (This study)	7199-7218
Badna 3R2	AAYKWBCCRCTWGCRTA	-do-	6880-6899
Badna 3R3	TTYCCDTYDTGTGYTCRAT	-do-	7353-7379

HE 33 Mini horizontal Submarine Unit) for 90 min, visualized and photographed using Alpha Imager (Alpha Innotech Corporation). The desired product on the gel was sliced and DNA was extracted using QIAquick gel extraction kit (Qiagen) as per manufacturer's instructions. The gel-extracted DNA was then ligated to a T-vector in presence of T4 DNA ligase using Instant TA cloning kit (Genei, Bangalore, India). The ligated plasmid was later introduced into competent *E. coli* cells using standard procedure (Sambrook and Russel, 2001). The recombinants were identified by PCR and restriction endonuclease digestion. The selected clones were sequenced at the automated sequencing facility at the AvasthaGen Grain Technologies, Bangalore, India.

Sequence comparison and phylogenetic analysis

BLAST programme (Altschul *et al*., 1997) was used to identify the related sequences from the GenBank database. The details of sequences and their accession numbers used for comparison are listed in Table 2. Multiple alignment was created in Clustal X and sequence phylograms were constructed by Neighbour hood joining boot strap method in Clustal X (version 1.83) and rooted trees were generated using TREE VIEW software (Page, 1996). RTBV was used as out group member. Pair wise percent identity was determined using the Bio Edit programme

Detection of the virus through PCR

Standardization of total DNA isolation protocol from black pepper: For the isolation of good quality template DNA for use in PCR, the protocol described by de Silva (2002) *et al.,* was used with slight modifications. In the modified protocol 100 mg of leaf tissue from infected plants were ground in 500µl of CTAB buffer [100mM Tris HCl (pH 8), 4mM EDTA (pH 8), 1.4 M NaCl, 2% CTAB, 1% PVP, 0.5% β-mercaptoethanol] and incubated at 65 ^{0}C for 30 min. The homogenate was later allowed to cool to room temperature and extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged at 2500g, for 10 min at room temperature. The supernatant was collected and 0.1 volume of 10% CTAB was added. This mixture was later re-extracted using chloroform: isoamyl alcohol (24:1) followed by centrifugation at 2500g for 10 min at room temperature. The supernatant was collected and to this 0.1 volume of 3 M sodium acetate (pH 5.2) and an equal volume of isopropanol were added and incubated in ice for 30 min for the DNA to precipitate. The DNA was pelletized by centrifugation at 7000g for 15 min at 4 ^{0}C, washed in 70% ethanol, air dried and dissolved in 100µl sterile distilled water. The DNA quantity was estimated by spectrophotometer.

Standardization of PCR for detection: A known infected Calicut isolate of PYMoV was used for standardization. The primer pair for PCR detection of the badnavirus was designed based on the ORF I sequence of badnavirus infecting black pepper in India. The PCR reaction contained 1x PCR buffer, 2.5mM $MgCl_2$, 200µM dNTPs, 50ng each of forward (SCBVR1) and reverse (Badna 1R) primers, 1.5 Units of *Taq* polymerase, template DNA and sterile water to a final volume 50µl. The thermal cycler was programmed for initial denaturation at 94 ^{0}C for 5 min, followed by 34 cycles of denaturation at 94 ^{0}C for 30 s, annealing at 56 ^{0}C for 1 min, synthesis at 72 ^{0}C for 1 min and a final extension for 10 min at 72 ^{0}C. The PCR products were analyzed on 1% agarose gel. To estimate the optimum template volume needed for PCR, DNA extracted from 200mg of leaf tissue was dissolved in 200µl of water and used in PCR at different volumes ranging from 0.01µl to 30µl. (0.01, 0.05, 0.1, 0.5, 1, 2, 3, 4, 5, 10, 15, 20, 25,30 and 35µl). In order to examine the reliability of the PCR for screening the plants for the presence of badnavirus in nursery and field plants, DNA was isolated from various test plants (representing different geographical regions) using the protocol described above and PCR was performed using 5 µl template DNA, along with a known healthy (negative control) and infected (positive control) samples.

RESUTLS

Amplification, cloning and sequencing

Among the thirteen combinations of primers tested, two primer pairs were found successful in amplifying the portion of ORF I and ORF III of the *Badnavirus* infecting black pepper. The primer pair SCBVR1 and Badna 1R, amplified a fragment of ~700 bp corresponding to portion of ORF I, while the primers Badna 3F2 and Badna 3R1 amplified of a fragment of ~600 bp corresponding to portion of ORF III in all the isolates used in the analysis; however no such bands were observed in healthy samples. These PCR products were cloned and their nucleotide sequence was determined. The GenBank accession numbers for each of these sequences is given in Table 2.

Sequenced region of ORF I of Calicut isolate of the virus contained 694 nucleotides. Comparative sequence analysis of this ORF I region with other badnaviruses showed that the black pepper *Badnavirus* shared maximum sequence identity (98% at nucleotide and 97% at the amino acid level) with PYMoV infecting black pepper reported from Sri Lanka. In contrast, the black pepper *Badnavirus* (Calicut isolate) showed an identity of only 18 to 30% with other badnaviruses used in the comparison

(Table 3). Phylogenetic tree constructed using these sequences also revealed that among badnaviruses; black pepper isolate from India is most closely related to PYMoV forming a single cluster that is well separated from other badnaviruses (Fig. 1).

Sequenced region of ORF III of Calicut isolate contained 597 nucleotides. Comparative analyses of this region with other badnavirus isolates showed that the identity of black pepper *Badnavirus* ranged from 47 to 67 % with different badnaviruses at the nucleotide level while identity at the amino acid level ranged from 31 to 69% (Table 3). The conserved motif YILDDILV present in all badnaviruses was found in the present isolate also. As sequence data for ORF III region sequence of PYMoV was not available, it could not be used in the comparison.

The identities in the cloned ORF III region among strains within a badnavirus species was >73 % at the nucleotide level and >76% at amino acid level where as between distinct species identity at nucleic acid level ranged from 33 to 67 % and 31 to 70 % at the amino acid level (Table 3). Based on this criterion, black pepper isolate of badnavirus from India does not belong to any of the species used in the comparison. Phylogenetic tree based on both nucleotide and amino acid also showed distinctive nature of all badnavirus isolates from black pepper (Fig. 1, 2) that are well separated from others. Thus based on the phylogenetic and sequence identities

Table 2. List of badnaviruses and their accession numbers used for analysis

Virus	GenBank accession number	Designation used
Piper yellow mottle virus (Idukki isolate)	DQ836228, DQ836229 (This study)	PYMoV-IDK
Piper yellow mottle virus (Calicut- isolate)	DQ836226, DQ836227 (This study)	PYMoV-CLT
Piper yellow mottle virus (Coorg isolate)	EU009725, DQ836232, (This study)	PYMoV-CRG
Piper yellow mottle virus (Sri Lanka isolate)	AJ626981	PYMoV-SRL
Banana streak GF virus	AY493509	BSV-GF
Banana streak Mysore virus	AY805074	BSV-Mys
Banana streak virus isolate GD	DQ451009	BSV-GD
Banana streak OL virus	AJ002234	BSV-OL
Banana streak virus strain Acuminata Vietnam	AY750155	BSV-AcVnm
Banana streak virus Acuminata Yunnan	DQ092436	BSV-Yun
Cacao swollen shoot virus	AJ534983	CSSV-TN
Cacao swollen shoot virus	AJ609019	CSSV-PEKI
Cacao swollen shoot virus	AJ609020	CSSV-N1A
Cacao swollen shoot virus	AJ781003	CSSV-TL
Citrus yellow mosaic virus	AF347695	CYMV
Commelina yellow mottle virus	X52938	ComYMV
Dioscorea alata bacilliform virus	X94575	DBV-L85-36
Dioscorea alata bacilliform virus	X94576	DBV-L85-20
Dracaena mottle virus	DQ473478	DMV-Ch
Kalanchoe top-spotting virus	AY180137	KTSV
Sugarcane bacilliform IM virus	AJ277091	SCBV-IM
Sugarcane bacilliform Mor virus	M89923	SCBV-Mor
Taro bacilliform virus	AF357836	TaBV-NG
Bougainvillea spectabilis chlorotic vein-banding virus	AY532653	BSCVBV
Canna streak virus	AJ810079	CSV-A
Pineapple bacilliform virus	Y12433	PBV
Rubus yellow net virus	AF468454	RYNV
Spiraea yellow leaf spot virus	AF299074	SYLSV
Stilbocarpa mosaic bacilliform virus	AF478691	SMBV
Yucca bacilliform virus isolate GU-1	AF468687	YBV-GU1
Yucca bacilliform virus isolate GU-2	AF468688	YBV-GU2
Rice tungro bacilliform virus	AF220561	RTBV

of portion of ORF I and ORF III it was concluded that *Badnavirus* infecting black pepper in India is a strain of PYMoV.

Sequence variability

In order to understand whether variability exists in the PYMoV infecting black pepper under different geographical areas in India, sequences of a portion of ORF I and III of PYMoV isolates collected from three different geographical regions were amplified, cloned and sequenced as described earlier. The number of nucleotides in the sequenced region of ORF I varied from 694 to 710 in different isolates. At the nucleotide level, the identity among the three isolates and the Sri Lankan isolate was >96%. The sequence phylogram constructed based on nucleotide sequences also showed close clustering of Coorg (PYMoV-CRG), Calicut (PYMoV-CLT) and Idukki (PYMoV-IDK) isolates with Sri Lankan PYMoV (PYMoV-SRL) (Fig. 1). At the amino acid level the identity among the Indian isolates of PYMoV ranged from 93 to 98% while with the Sri Lankan isolate identity ranged from 94 to 98%.

The number of nucleotides in the sequenced region of ORF III varied from 596 to 600 in different isolates. Pair wise comparison of sequences revealed an identity ranging from 95 to 98 % at the nucleotide level and 96 to 98 % at the amino acid level among the isolates (Table 3). The badnavirus conserved motif YILDDILV was found in all the isolates. Phylogenetic tree constructed using both nucleotide and amino acid sequences of the ORF III region confirmed results of sequence alignment and showed distinctive nature of all black pepper isolates from India (PYMoV-CRG, PYMoV-CLT and PYMoV-IDK) as it did not cluster with any of the distinct badnavirus species used (Fig. 2). The phylogenetic tree also depicted close clustering of all strains of a species such as, *Sugarcane bacilliform virus* (SCBV), *Cacao swollen shoot virus* (CSSV).

Detection of the virus through PCR

The concentration of total DNA isolated from black pepper varied (126 to 137 ng/µl) in different preparations. When this total DNA at different dilutions along with primers SCBVR1 and Badna 1R were used for the detection

Table 3. Sequence identities of selected isolates of badnaviruses used for comparison. The values above diagonal line represent the nucleotide identitity in the 700bp region of ORF I and the values below diagonal line represent the amino acid identitity in the 200 amino acid region of ORF III. (A:*SCBV-IM*, B:*SCBV-Mor*, C:*BSV-AcVnm*, D:*BSV-Yun*, E:*BSV-Mys*, F:*BSV-GD*, G:*BSV-OL*, H:*CSSV-PEKI*, I:*CSSV-NIA*, J:*CSSV-TN*, K:*CYMV*, L:*.DMV-Ch*, M:*TaBV-NG*, N:*DBV-L85-36*, O:*DBV-L85-20*, P:*PYMoV-IDK*, Q:*PYMoV-CLT*, R: *PYMoV-SRL*, S:*PYMoV-CRG*, T:*BSV-GF*, U:*ComYMV*, V:*KTSV*, W:*RTBV*, X:*CSSV-TL*, NA: Not available)

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
A	1	71	38	0	33	46	40	40	48	48	50	32	37	40	39	41	30	30	28	30	34	40	25	17
B	91	1	42	35	42	40	41	41	27	27	26	26	27	26	27	27	24	24	24	24	37	34	29	21
C	53	57	1	32	39	47	44	43	28	28	26	28	25	24	27	27	27	27	26	27	39	37	30	20
D	60	63	68	1	72	46	47	48	23	23	22	25	22	22	22	22	18	18	19	19	35	30	27	17
E	61	62	71	74	1	53	59	61	31	30	30	31	29	28	30	29	22	22	23	23	42	43	32.3	22
F	61	65	90	78	74	1	51	50	26	25	25	28	25	25	28	27	25	25	24	25	37	34	32	20
G	61	65	90	78	74	1	1	94	29	28	29	28	27	26	29	29	24	24	24	24	38	39	33	23
H	59	60	56	65	61	65	65	1	29	29	30	29	27	26	29	30	24	24	24	24	40	39	32	23
I	57	58	57	63	59	63	63	97	1	97	81	59	45	49	48	48	21	21	21	21	32	29	24	26
J	58	59	53	64	59	62	62	94	91	1	81	59	45	48	48	48.6	21	21	20	21	31	29	24	25
K	59	60	59	65	67	67	66	69	67	69	1	58	46	46	47	47	19	19	19	19	30	30	22	25
L	59	58	59	66	64	67	67	60	58	61	64	1	44	46	47	47	19	19	19	19	32	29	24	25
M	62	64	53	62	60	61	61	63	61	63	60	61	1	51	42	42	18	18	19	19	29	26	24	23
N	27	28	31	34	33	34	34	30	30	30	31	32	30	1	43	44	18	18	18	18	33	27	28	27
O	28	29	31	35	33	34	34	30	30	30	31	31	30	96	1	95	20	20	20	20	30	28	23	26
P	62	61	62	66	69	70	70	63	61	63	68	65	65	31	32	1	21	21	21	20	29	27	24	26
Q	61	60	62	66	69	69	69	63	61	63	68	65	65	31	31	97	1	98	96	96	23	20	24	17
R	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	96	96	23	21	24	17
S	61	61	62	66	70	70	70	63	61	63	67	66	66	32	32	96	96	NA	1	96	22	20	24	17
T	60	62	64	70	65	73	73	58	56	56	62	63	61	30	30	66	66	NA	65	1	22	21	25	17
U	63	64	58	62	64	66	66	59	57	57	63	64	61	28	29	64	63	NA	64	61	1	36	33	20
V	56	57	67	77	71	76	76	59	57	58	65	66	60	33	34	66	66	NA	66	67	60	1	29	20
W	45	45	38	41	43	44	44	44	42	43	44	43	47	23	23	41	40	NA	40	43	41	42	1	24
X	58	60	57	65	61	66	66	81	79	84	69	63	63	30	31	65	66	NA	66	59	57	61	40	1

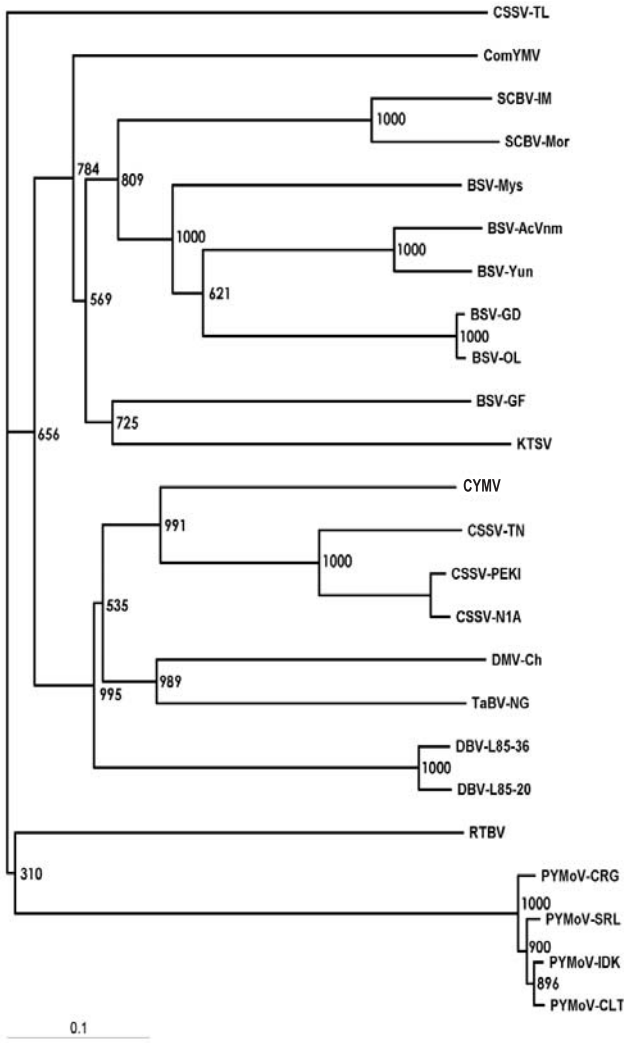

Fig. 1: Phylogram illustrating phylogenetic relationships based on the multiple alignments of ORF I nucleotide sequences of 19 distinct badnavirus isolates and black pepper isolates from India (PYMoV- CLT, PYMoV- IDK, PYMoV- CRG). Designations used for each of the isolates are listed in Table 2. Bootstrap values are presented at the nodes. RTBV was used as out group member

of the virus in PCR, a product of expected size (~700 bp) was seen in all the template volumes used except in 30µl. The volume of template required for the better amplification of the virus was found to be in the range 0.5µl to 15µl (corresponding to 65 to 1650 ng of total DNA). In order to test the utility of PCR method standardized, 5 µl of the template DNA isolated from 230 plants of black pepper (which included field plants, nursery plants and seedlings from different regions) were subjected to PCR along with known positive and negative controls. The virus was successfully detected in symptomatic as well as asymptomatic plants (Fig. 3). Of the 230 plants, 84 were found positive for PYMoV infection. Among the PYMoV positive plants 61 showed symptoms while rest did not show any visible external symptoms.

DISCUSSION

The current study based on the sequences of portion of ORF I and ORF III identified the *Badnavirus* infecting black pepper in India as a strain of *Piper yellow mottle virus* (PYMoV) that is reported to infect black pepper in south East Asian countries such as Indonesia, Malaysia, The Philippines, Sri Lanka and Thailand (Lockhart *et al*., 1997; de silva *et al*., 2002; Suastika *et al*., 2005). This is the first report on the identification and sequence variability analysis of PYMoV infecting black pepper in India. In general ORF I of badnaviruses contain 431 to 602 nucleotides and its function in many badnaviruses

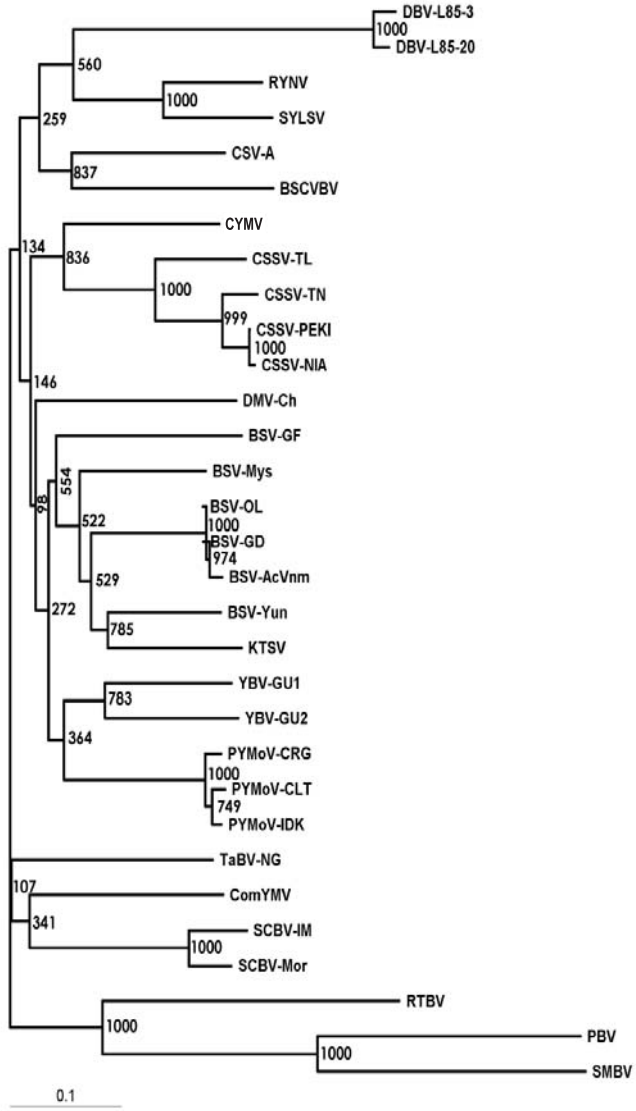

Fig. 2: Phylogram illustrating phylogenetic relationships based on the multiple alignments of the portion of ORF III amino acid sequences of 27distinct badnavirus isolates and black pepper isolates from India (PYMoV- CLT, PYMoV- IDK, PYMoV- CRG). The designations used for each isolate is given in Table 2. Boot strap values are presented at the nodes. RTBV was used as out group member

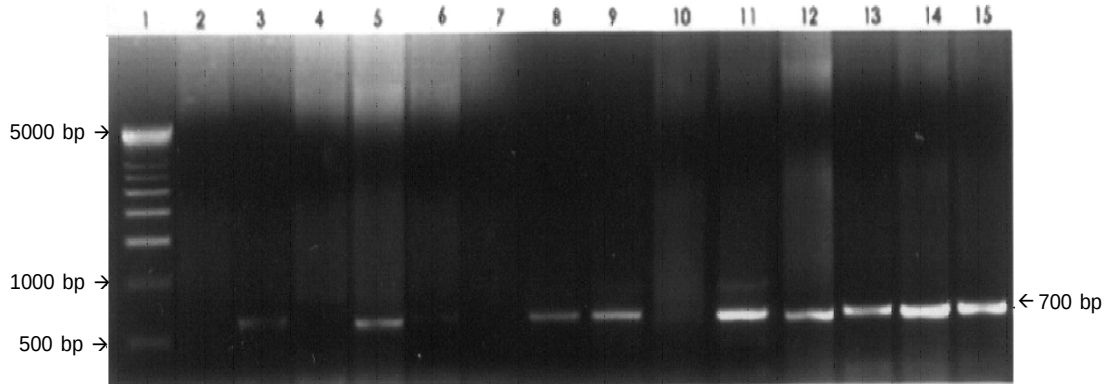

Fig. 3: Detection of Badnavirus *in field plants. Lane 1. 500bp Marker; lane 2: negative control (healthy black pepper); lanes 3-14: test plants; lane 15: positive control (*Badnavirus *infected black pepper)*

including PYMoV is not yet known, while ORF III range from 3387 to 5825 bp in different badnaviruses and is known to code for a polyprotien which is later cleaved in to many functional proteins (Hohn and Futterer, 1997). The ORF III region cloned in the present study showed homology with the reverse transcriptase protein.

Intra specific level variations are known in badnavirus species such as SCBV (Geijskes *et al*., 2002), *Taro bacilliform virus* (TaBV) (Yang *et al*., 2003), ComYMV (Medberry *et al*., 1990), *Diascorea alata bacilliform virus* (DBV) (Briddon *et al*., 1999) and CSSV (Muller *et al*., 2005). Among other distinct badnaviruses, PYMoV was found closer to BSV-GF, BSV-Mys, BSV-OL and SCBV. Serological relationships of PYMoV with BSV and SCBV have been reported (Lockhart *et al.,* 1997; Bhat *et al*., 2003). In India black pepper is mostly grown as mixed crop with arecanut, banana, coconut, cocoa, coffee, tea and occurrence of badnaviruses such as BSV, SCBV, *Citrus yellow mosaic virus* (CYMV) are reported (Ahlawat *et a1*., 1996; Viswanathan *et al*., 1996). Though variations in the symptoms were observed under field conditions in stunted disease affected black pepper, the three such isolates used in the present study did not show much variation in the sequenced region of ORF I and III. However only complete genome sequencing of isolates would reveal the diversity among different isolates, hence a large number of isolates representing different cultivars and region need to be carried out to understand the diversity in PYMoV. Further, a few badnaviruses are known to integrate their genome into their host genome and whether this kind of integration occur in PYMoV-black pepper system need to be studied.

In most cases stunted disease affected plants do not show visible symptoms throughout the year. Masking of symptoms is observed especially during monsoon period (June to September) under Indian conditions. ELISA based methods also failed to provide fool-proof detection of PYMoV owing to low titre of the virus in plants (de Silva *et al*., 2002; 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 it is essential to use sensitive techniques such as PCR. The protocol standardized for DNA isolation and PCR successfully detected the PYMoV in infected pepper plants (with or without visible symptoms) in a wide range of DNA concentration ranging from 65 to 1650ng. The method can be used for large scale indexing of black pepper plants to identify PYMoV free plants.

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