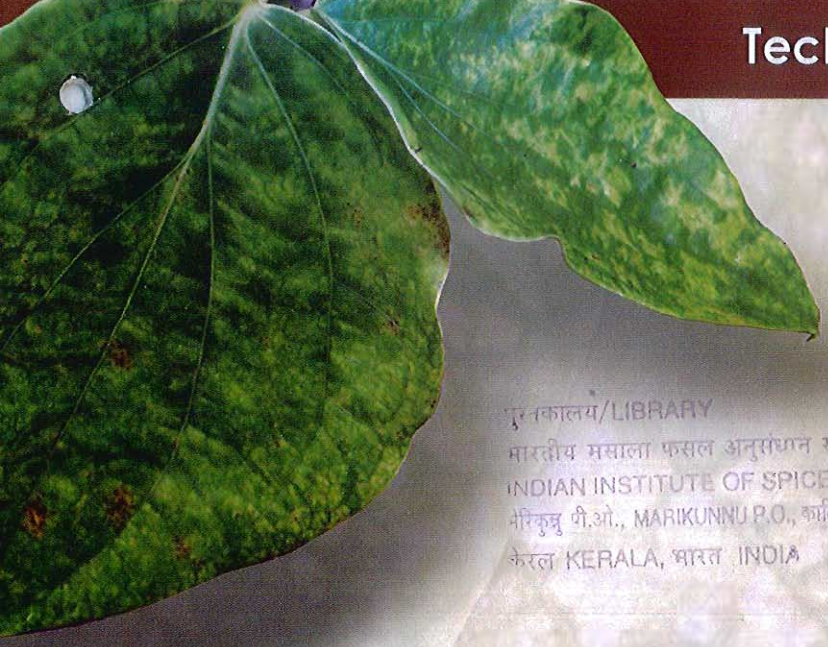
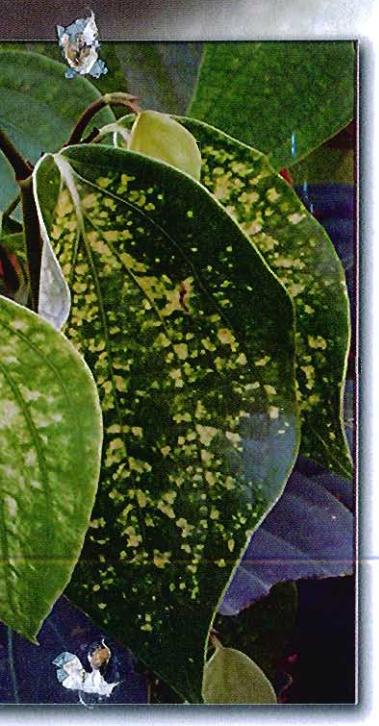
Technical Bulletin





Viral Disease and its Management in Black Pepper

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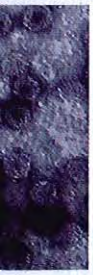

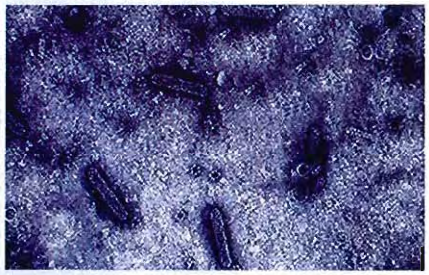

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INTRODUCTION

Black pepper (*Piper nigrum* L.) which originated in the tropical evergreen forests of Western Ghats of India is used for a variety of purposes including in medicine. It constitutes an important component of culinary seasoning of universal use and is an essential ingredient of numerous commercial foodstuffs. Although wilt diseases caused by oomycetes and nematodes continue to be major diseases infecting black pepper, of late viral disease caused by viruses is becoming increasingly important due to its systemic nature and yield loss. Virus induced symptoms referred differently like dog's ear, mosaic, little leaf, wrinkled leaf and stunted disease have been reported from several black pepper growing countries such as Brazil, India, Indonesia, Malaysia, Philippines, Sri Lanka and Thailand and has been reviewed recently by Bhat (2008).

DISTRIBUTION, INCIDENCE AND YIELD LOSS

The disease is distributed in all black pepper growing countries of South East Asia and Brazil. In India, Bhat et al. (2005c) reported a disease incidence ranging from 0% to 100% in different plantations. The incidence of the disease was highest in Wyanad District (45.4%) followed by Idukki District (29.4%) in Kerala (Table 1). In Karnataka, Kodagu District (14.9%) had the highest incidence of the disease followed by Hassan District (5.2%). In general both incidence and severity of disease was higher in black pepper plantations located at high altitudes. The yield loss due to the disease was studied in a fixed plot at Polibetta (Kodagu, Karnataka). Based on severity, infected plants were stratified into four groups and yield was recorded in relation to canopy size of 10 to 12 year old Panniyur 1 plants. In the virus infected plants, loss in yield varied from 16% to 85% (IISR, 2005). All cultivars and improved varieties including hybrids were susceptible to the disease under natural conditions. More than 1000 cultivated accessions of black pepper screened against the virus at Indian Institute of Spices Research (IISR), Kozhikode, showed susceptible reaction. Vines of all ages raised on all kinds of standards were also found affected by the disease.

SYMPTOMS

A wide range of symptoms are observed on infected vines under field conditions. Mosaic, mottling, leaf deformation and stunting of whole plant are the most visible symptoms in the field (Fig. 1). The initial symptoms of the disease include chlorotic specks, vein clearing, mosaic and yellow mottling (Fig.1). Severe symptoms sometimes develop sporadically on flushes of new growth, while other leaves showed milder symptoms or were symptomless. The infected vine produces spikes with short spike and poor filling leading to yield reduction. In severe cases, the leaves become abnormally narrow and appear sickle shaped (Fig. 1). The internodes of vines become abnormally short leading to stunting of plants and the affected branches give a typical witches broom appearance in advanced stages (Paily et al., 1981; Lockhart et al., 1997; Sarma et al., 2001; de Silva et al., 2002; Bhat et al., 2003). Sometimes depending on the season, growth stage and other factors, the disease affected plants do not produce any visible external symptoms. This kind of masking of symptoms in certain affected plants may be seen during monsoon and winter months while symptoms are best exhibited during summer months. Further, symptoms are exhibited prominently in certain cultivars like Karimunda, while symptoms are rarely seen in varieties like Panniyur 1, Panniyur 5 and Panchami and, some of the plants in these varieties remain symptomless for many years. These virus infected symptomless plants can also act as source for secondary spread of the virus in the field (Bhat et al., 2012).

CAUSAL VIRUSES

Though the viral nature of the disease was estab-

Table 1. Incidence of viral disease of black pepper in Karnataka and Kerala

State/District	Disease incidence	
	Range (%)	Mean (%)
Karnataka		
Dakshina Kannada	0	0
Hassan	0-20	5.2
Madikeri	0-78	14.9
Uttara Kannada	0-2	0.4
Kerala		
Idukki	0-78	29.4
Kannur	2-42	19.5
Kasaragod	0-53	18.9
Kozhikode	0-33	10.7
Wyanad	13-83	45.4

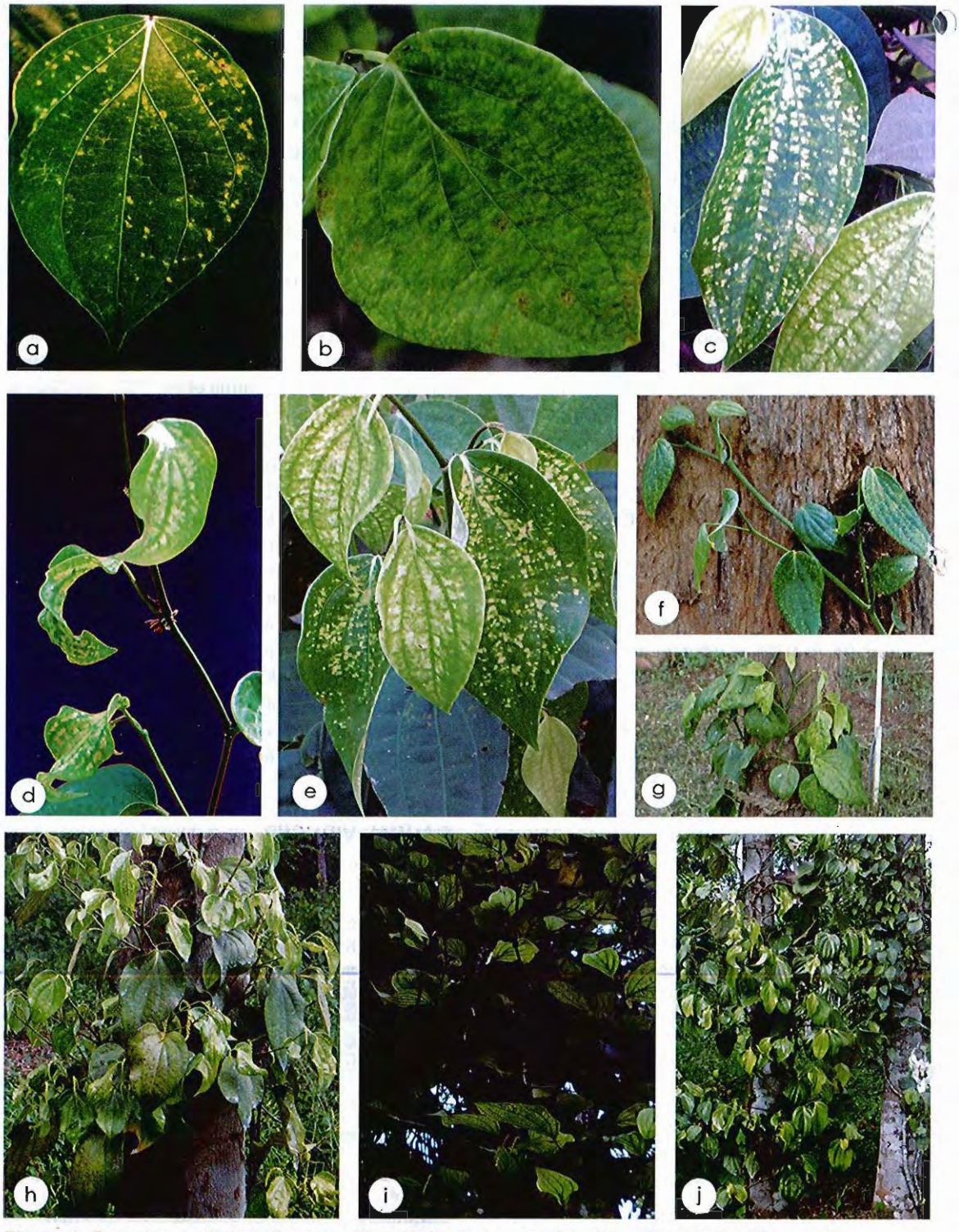

Figure 1. Symptoms of viral disease affected black pepper. (a) chlorotic specks (b) mosaic (c) mottling (d) deformation and reduction in leaf size (e, f, g) symptoms on lateral branch (h, I, j) infected vines showing different levels of infection

lished as early as 1959, the identity of the causal virus(es) remained uncertain for many years. Non enveloped bacilliform like particles resembling those of badnaviruses were observed by electron microscopy in partially purified extracts of symptomatic plants (Lockhart et al., 1997). They reported a new mealybug transmitted badnavirus, namely *Piper yellow mottle virus* (PYMoV) as the cause of the disease in Malaysia, Thailand, Philippines and Sri Lanka. Subsequently, occurrence of PYMoV was reported on black pepper from India (Bhat et al., 2003; Hareesh and Bhat, 2008). In addition to PYMoV, association of another virus namely, *Cucumber mosaic virus* (CMV) in diseased black pepper plants was reported from India (Sarma et al., 2001; Bhat et al., 2005a). At present occurrence of CMV is also reported with diseased black pepper in all pepper growing countries in the South East Asia and Brazil. Thus association of either one or both the viruses are seen in affected plants. The intensity of the disease was more when both the viruses are associated. Plantations with high incidence and severity of the disease were invariably were infected by both the viruses (Bhat, 2008).

Cucumber mosaic virus (CMV)

Characterization

CMV, the type species of the genus, *Cucumovirus* in the family of Bromoviridae, is one of the most widespread plant viruses in the world. Its genome consists of three plus-sense single stranded RNAs plus a fourth subgenomic RNA. RNAs 1 and 2 are associated with viral genome replication, while the RNA 3 encodes for movement protein and coat protein. CMV has an extensive host range infecting over 1000 species in more than 85 plant families (Palukaitis et al., 1992). The numerous strains of CMV have been classified into two major subgroups (subgroup I and II) on the basis of serological properties and nucleotide sequence homology. The subgroup I has been further divided into two groups (IA and IB) by phylogenetic analyses.

The CMV infecting black pepper was mechanically transmitted and propagated on *Nicotiana benthamiana* and *N. glutinosa*. Symptoms appeared within 7-10 days in these inoculated plants. Symptoms on *N. benthamiana* included severe puckering, mosaic, mottling and downward curling of leaves while in *N. glutinosa*, vein clearing followed by mosaic and yellow mottling with slight curling at leaf margins were the prominent symptoms (Bhat et al., 2004). Purification of CMV infecting black pepper was reported by de Silva et al. (2001) and Bhat et al. (2004). A 260/280 ratio of the purified virus was 1.65 and yield of virus obtained per 100 g of tissue varied from 1 to 4 mg depending on the harvest time after inoculation. Electron microscopy of negatively stained purified preparations revealed the presence of isometric particles of about 28 nm diameter (Fig. 2) (Bhat et al., 2004).

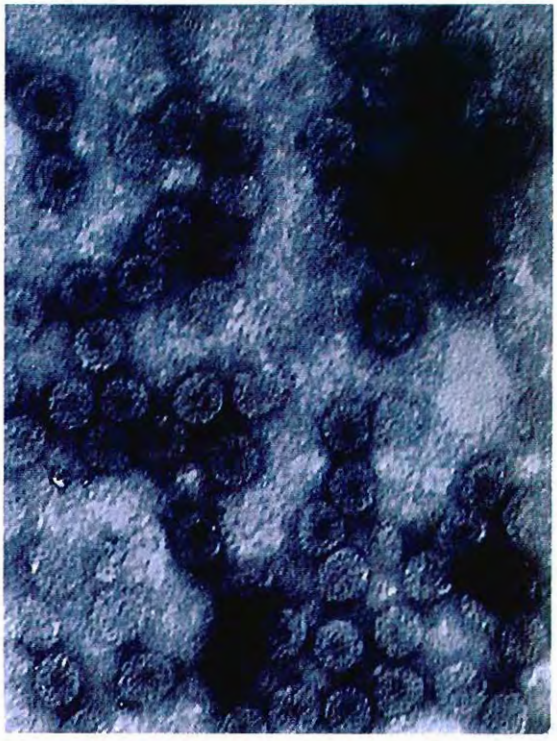

Figure 2. Electron micrograph of purified preparation of *Cucumber mosaic virus* from black pepper

The coat protein gene of black pepper isolate of CMV comprised of 657 nucleotides encoding a protein of 218 amino acids. Sequence analyses showed that the amino acid sequence identity with members of subgroup I was 92% - 99% while that with members of subgroup II was from 77% to 79%. On the basis of sequence homology and phylogenetic relationship studies, it is concluded that CMV infecting black pepper in India is a strain of CMV belonging to subgroup IB (Fig. 3) (Bhat et al., 2005a). As CMV is known to infect other *Piper* species such as *P. longum* (Indian

long pepper) and *P. betle* (betel vine), coat protein gene of CMV from these hosts were also cloned and sequenced. Pair wise comparison showed that the CP gene sequence of CMV from betel vine and Indian long pepper were highly conserved with 100% sequence identities both at nucleotide and amino acid levels (Hareesh et al., 2006). Further, CMV isolates from *P. betle* and *P. longum* were closer to CMV isolates of subgroup IB infecting banana and black pepper. In order to understand the diversity of the CMV infecting different *Piper* spp., coat protein gene of CMV isolate infecting black pepper, betel vine and Indian long pepper from Kozhikode and Kasargod districts of Kerala were cloned, sequenced and compared. Sequence analysis showed a very high level of sequence identity (>99%) both at nucleotide and amino acid level among all the isolates.

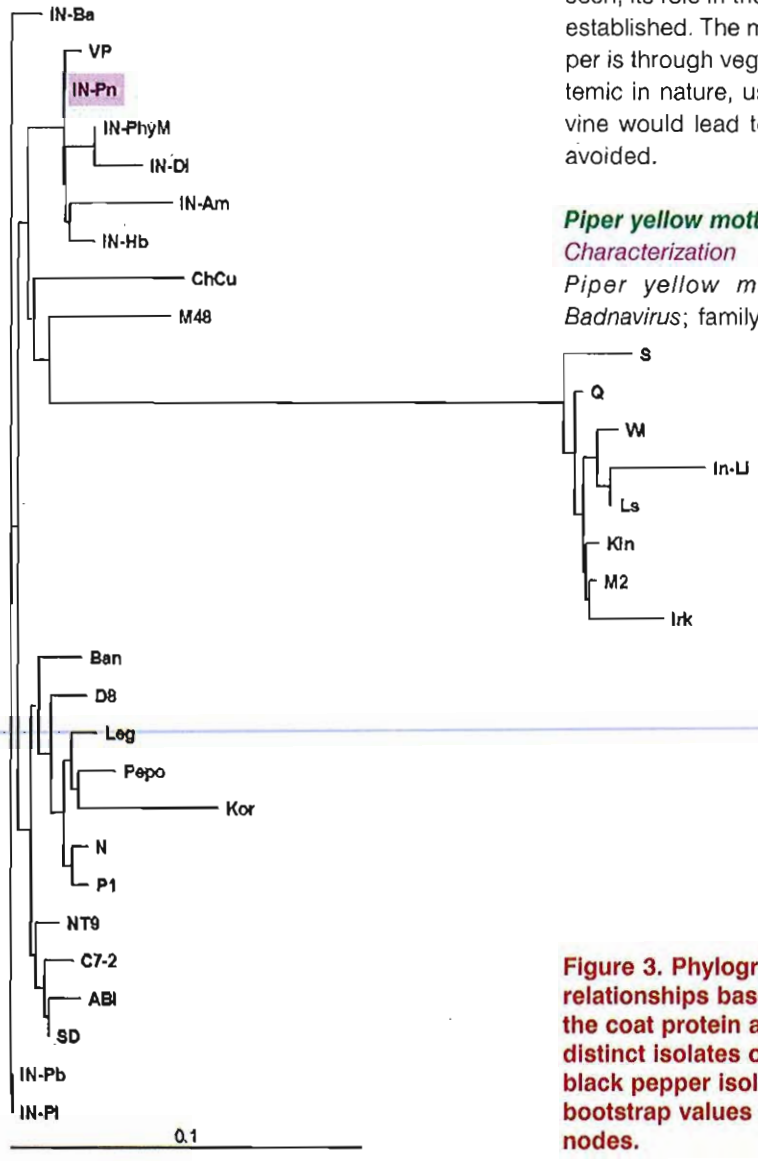

Figure 3. Phylogram illustrating phylogenetic relationships based on multiple alignments of the coat protein amino acid sequences of distinct isolates of *Cucumber mosaic virus* and black pepper isolate of CMV (IN-Pn). The bootstrap values are shown at the individual nodes.

Transmission

CMV from black pepper could be easily transmitted mechanically to several cucurbitaceous and solanaceous hosts (de Silva et al., 2001; Sarma et al., 2001). In general, CMV is known to have a very wide host range infecting several plant species and spreads in nature through aphids in a non-persistent manner. Although colonization of aphids on black pepper is seen, its role in the transmission of CMV is yet to be established. The major spread of CMV in black pepper is through vegetative means. Viruses being systemic in nature, use of stem cuttings from infected vine would lead to spread of virus and has to be avoided.

Piper yellow mottle virus (PYMoV)

Characterization

Piper yellow mottle virus (PYMoV) (genus: *Badnavirus*; family: Caulimoviridae) has non-envel-

oped bacilliform shaped virions with ds DNA genome of about 7.5 kb. The virus is not mechanically transmitted either from black pepper to black pepper or to other *Piper* spp. PYMoV particles were purified from infected leaves of black pepper (Lockhart et al., 1997; de Silva et al., 2002; Bhadramurthy et al., 2005). The electron microscopy of purified preparation showed non-enveloped bacilliform particles of 28-32 nm in width and 115-135 nm in length (Fig. 4). In ultra thin sections of black pepper, virions of PYMoV were observed in the cytoplasm but not in the nucleus of infected cells.

Figure 4. Electron micrograph of purified preparation of *Piper yellow mottle virus* from black pepper

Immunosorbent electron microscopy (ISEM) (Lockhart et al., 1997), F(ab)2 indirect ELISA (de Silva et al., 2002), direct antigen coated (DAC) ELISA (Bhat et al., 2003) and double antibody sandwich (DAS)-ELISA (Bhadramurthy et al., 2005) were used to determine the serological relationship of PYMoV. In ISEM tests, PYMoV antiserum trapped *Banana streak virus* (BSV) but not other badnaviruses tested such as *Commelina yellow mottle virus* (ComYMV), *Kalanchoe top spotiing virus* (KTSV) and *Sugarcane bacilliform virus* (SCBV) while in reciprocal test, PYMoV particles were trapped by antiserum against SCBV but not to antisera to BSV, ComYMV, *Cocao swollen shoot virus* (CSSV), *Dioscorea bacilliform virus* (DBV), KTSV or *Rice tungro bacilliform virus* (RTBV) (Lockhart et al., 1997). Using DAC-ELISA, Bhat et al. (2003) also reported serological relationship of PYMoV with BSV and SCBV.

Using degenerate primers to the conserved regions present in several badnavirrus isolates, Lockhart et al. (1997) amplified a 450 bp region corresponding to reverse transcriptase gene through PCR. Based on nucleotide sequence comparison of this region, PYMoV was found to be closely related to mealybug transmitted badnaviruses such as BSV, ComYMV and SCBV than to RTBV and more distantly related to members of caulimoviruses. Later de Silva et al. (2002) reported amplification and sequencing of a 700 bp region of open reading frame (ORF) I of a Sri Lankan isolate of PYMoV through PCR. Recently Hareesh and Bhat (2008) reported amplification, cloning and sequencing of 700 bp region of ORF I and 600 bp region of ORF III of an Indian isolate of PYMoV. Comparison of PYMoV isolates from Sri Lanka and India showed an identity of 98% while it was only 18% to 30% with other distinct badnavirus species in the ORF I sequence. Sequenced region of ORF III of Indian isolate contained 597 nucleotides. Comparative analyses of this region with other badnavirus isolates showed that the identity of PYMoV ranged from 47% to 67% with different badnavirus species at the nucleotide level while identity at the amino acid level ranged from 31% to 69%. The conserved motif YILDDILV present in all badnaviruses was also found in PYMoV (Hareesh and Bhat, 2008). Phylogenetic tree constructed using the sequences also revealed that among badnaviruses, PYMoV isolates formed a separate cluster that was well separated from other badnaviruses (Fig. 5). Based on the sequence of portion of ORF I and ORF III of *Badnavirus* infecting betel vine and Indian long pepper, they were identified as strains of PYMoV. Recent studies on full genome sequencing of an isolate of PYMoV from India showed that it is about 7500 nucleotides long with four ORFs. (Deeshma and Bhat, unpublished).

Studies on the badnavirus infecting banana, taro, cacao and yam have shown the existence of high variability among virus isolates and occurrence of more than one badnavirus species infecting each of these crops. Complete genome sequencing of several isolates of PYMoV representing different agro-climatic regions and countries is needed to understand the existence of variability. A few of the pararetroviruses such as BSV, *Tobacco vein clearing virus* (TVCV) and *Petunia vein clearing virus* (PVCV) are known to integrate their genome into the host chromosome (reviewed in Bhat, 2008). Whether such integration occurs in PYMoV-black pepper system need to be looked into.

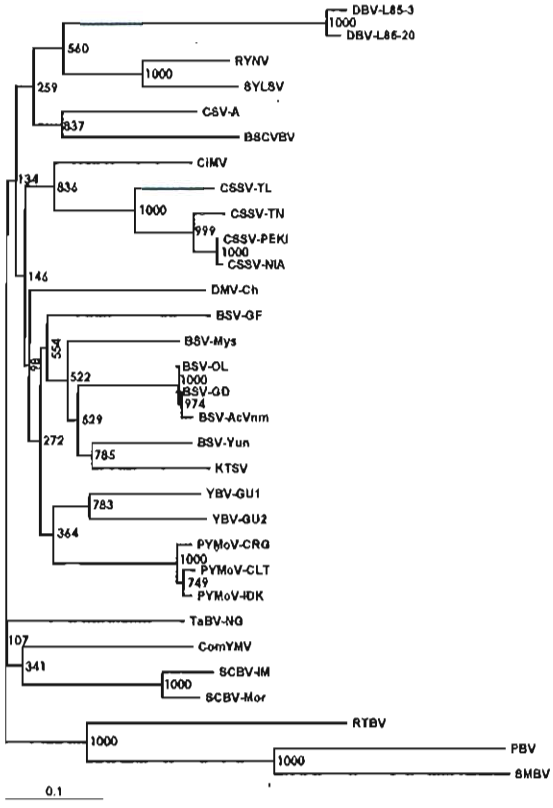

Figure 5. Phylogram showing phylogenetic relationships based on the multiple alignment of the 600 bp region of open reading frame (ORF) III amino acid sequence of 27 distinct badnavirus isolates and PYMoV isolates. Boot strap values are presented at the nodes. RTBV was used as out group member. Acronyms used for virus species: BSV-GF: *Banana streak GF virus*; BSV-OL: *Banana streak OL virus*; BSV-Mys: *Banana streak Mysore virus*; BSV-Yun: *Banana streak Acuminata Yunnan virus*; CSSV: *Cacao swollen shoot virus*; CYMV: *Citrus yellow mosaic virus*; ComYMV: *Commelina yellow mottle virus*; DBV: *Dioscorea alata bacilliform virus*; DMV: *Dracaena mottle virus*; KTSV: *Kalanchoe top-spotting virus*; SCBV: *Sugarcane bacilliform virus*; TaBV: *Taro bacilliform virus*; BSCVBV: *Bougainvillea spectabilis chlorotic vein banding virus*; CSV: *Canna streak virus*; PBV: *Pineapple bacilliform virus*; RYNV: *Rubus yellow net virus*; SYLSV: *Spiroea yellow leaf spot virus*; SMBV: *Stilbocarpa mosaic bacilliform virus*; YBV: *Yucca bacilliform virus*; RTBV: *Rice tungro bacilliform virus*.

Transmission

The primary spread of PYMoV occurs vegetatively through use of stem cuttings from infected vines for fresh planting. The virus can also be experimentally transmitted through grafting. However, the virus could not be transmitted to any other plant species tested either locally or systemically by mechanical sap inoculation. Recently seed transmission of the virus in four cultivars of black pepper was reported based on symptoms and PCR test on the seedlings raised from berries collected from infected plants (Table 2) (Hareesh and Bhat, 2010). This showed that seed can also serve as primary source of inoculum for the virus. However, seed transmission may not have much significance in the spread of PYMoV in plantations as black pepper is mainly propagated through vegetative means. But in breeding experiments this can cause considerable interference. Seeds can be infected when one of the parent is infected as there is a chance for the pollens and ovary to be infected.

The secondary spread of PYMoV occurs through different species of mealybug. Successful transmission of PYMoV from black pepper to black pepper was obtained with mealybug, *Planococcus citri* (Lockhart et al., 1997) while *P. citri* failed to transmit PYMoV from black pepper to other *Piper* species. In India, PYMoV was shown to be transmitted by two species of mealybug (*Ferrisia virgata* and *P. citri*) (Fig. 6) (Bhat et al., 2003; 2005b). Transmission of the virus was confirmed based on symptoms and PCR tests. Among the two species, *F. virgata* was found more efficient than *P. citri* in the transmission of PYMoV (Bhat et al., 2005b). In addition to mealybugs, de Silva et al. (2002) showed the involvement of a black pepper lace bug (*Diconocoris distanti*) in the transmission of PYMoV in Sri Lanka.

DETECTION AND DIAGNOSIS FOR VIRUSES

Symptoms

Though external symptoms are good criteria for detection, many a times depending on the season, growth stage and other factors, the disease can be difficult to identify or detect visually. So external symptoms can not be used as the sole criterion to identify healthy plants. There is a need to use sensitive techniques such as ELISA and PCR to detect the virus in mother plants so that such

Table 2. Seed transmission of *Piper yellow mottle virus* in black pepper

Variety	Symptomatological test			PCR test		
	No. of seedlings	No. of symptomatic seedlings	% symptomatic seedlings	No. of seedlings tested	No. of PCR positive seedlings	% infected seedlings
Sreekara	88	23	26	50	15	30
Subhakara	140	18	13	50	12	24
Panniyur -1	215	21	10	50	14	28
IISR-Shakthi	57	6	11	50	11	22

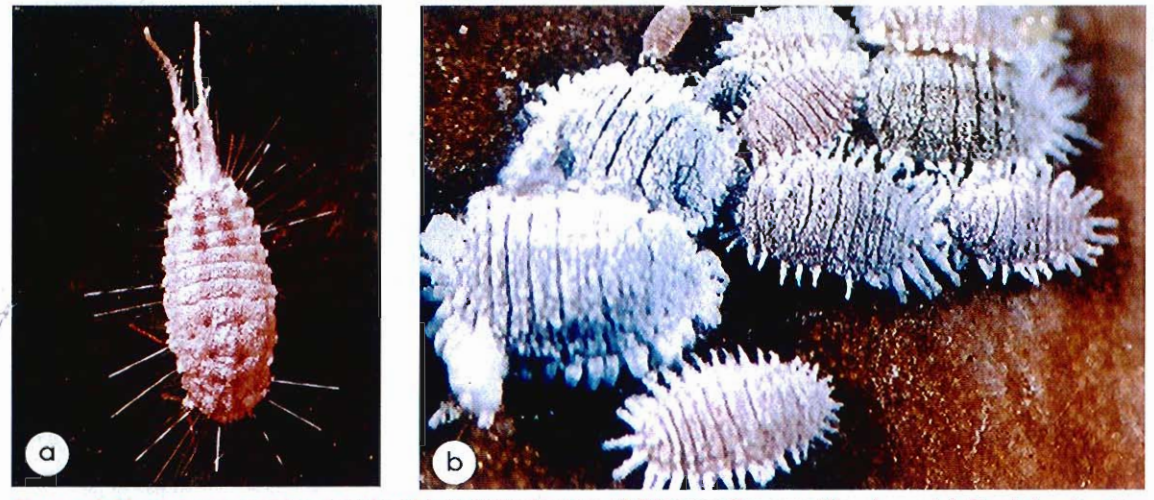

Figure 6 Mealybug species involved in transmission of *Piper yellow mottle virus*. (a) *Ferrisia virgata* (b) *Plancoccus citri*

contaminated source can be avoided for vegetative multiplication. Indicator plants can be used for the identification of CMV through sap inoculation under controlled conditions.

Enzyme linked immuno sorbent assay (ELISA)

Double antibody sandwich (DAS) ELISA based method for the specific detection of both CMV and PYMoV in black pepper samples have been reported. The method can be successfully used to detect the virus in black pepper plants, cuttings and seedlings for CMV (de Silva et al., 2001; Bhat et al., 2004) and PYMoV (Bhadramurthy et al., 2005). This could also be used in commercial nurseries to detect the presence of virus infection in stock (mother) plants and in preventing movement of infected plants to new areas. The method can be used to monitor spread and to identify resistance sources besides its use in the detection of virus in alternate crop and weed hosts.

The DAS-ELISA procedure detected CMV in diseased black pepper plants from different regions of Karnataka, Kerala and Tamil Nadu (Table 3) (Bhat et al., 2004). The varying OD values indicate varying virus concentration in samples. Further, within the diseased black pepper vine, the distribution of CMV was highly variable although the virus was found in all plant parts including stem and roots (Bhadramurthy et al., 2008). CMV infection was also detected from other naturally infected *Piper* species such as *P. chaba* Hunter, *P. colubrinum* Link and *P. longum* L. (Table 3). Of the 79 individual *P. colubrinum* plants tested, 13 were found positive for CMV infection (Table 3). Natural infection of CMV with all these three species indicates that they may act as potential source of inoculum for cultivated black pepper. While infected *P. chaba* and *P. longum* showed mosaic and stunting symptoms, no such symptoms were seen on *P. colubrinum* infected with CMV (Bhat et al., 2004).

Table 3. Detection of *Cucumber mosaic virus* by Double Antibody Sandwich ELISA* in *Piper* species from different regions

Piper nigrum isolate	Visual symptoms	A_{405} value$	*Piper* spp. isolate	Visual symptoms	A_{405} value$
Karnataka State			*P. chaba*		
Hassan District			Sample 1	MM, Y	0.33
Bantanahalli	M, LD, S	0.43	Sample 2	MM, Y	0.31
Belur	M, S	0.57	Sample 3	MM, Y	0.3
			Sample 4	MM, Y	0.21
Kodagu District			Sample 5	MM, Y	0.2
Balale	M	0.32			
Kodlipet	M, LD, S	0.78	*P. colubrinum*		
Mayamudi	M, S	0.37	Sample 1	–	3.92
Polibetta	M, S	0.41	Sample 2	–	3.89
			Sample 3	–	3.98
Udupi District			Sample 4	–	3.76
Idu	M, LD, S	0.68	Sample 5	–	2
			Sample 6	–	0.76
Kerala State			Sample 7	–	0.71
Idukki District			Sample 8	–	0.57
Adimali	M	0.75	Sample 9	–	0.51
Chakkuppalam	M, LD, S	0.22	Sample 10	–	0.35
Chottupara	M, LD, S	0.19	Sample 11	–	0.33
Muttom	M, S	0.15	Sample 12	–	0.3
Thookupalam	M, S	0.19	Sample 13		0.21
Vandiperiyar	M, S	0.15			
			P. longum		
Wyanad District			Sample 1	M, LD, S, B	0.53
Kenichera	M	0.49	Sample 2	M, S, B	0.46
Kuppadi	M, S	0.57	Sample 3	M, LD, S,	1.33
Pulpally	M, LD, S	2.01	Sample 4	M, LD, S, B	1.65
Vengapalli	M, S	0.78	Sample 5	M, LD, S, B	1.16
			Sample 6	M	0.22
Kozhikode District			Sample 7	M, LD, S, B	2.23
Peruvannamuzhi	M, LD, S	0.61	Sample 8	M, LD, S, B	1.03
			Sample 9	M, LD, S, B	1.98
Tamil Nadu State			Sample 10	M, LD, S, B	1.56
Coimbatore District			Sample 11	M, LD, S, B	0.99
Walayar	M	1.16	Sample 12	M, LD, S, B	1.98
Healthy black pepper	–	0	Healthy black pepper	–	0.04

B: Blisters on leaves; LD: Leaf distortion; M: Mosaic; MM: Mild mosaic; S: Stunting of the plant; Y: Yellowing; – : no visual symptoms

*Antigen and conjugate were used at 1:5 and 1:500 dilutions, respectively.

$ Average of three replications, 1 h after addition of substrate.

DAS-ELISA for detection of PYMoV in black pepper samples collected from different regions of India was reported though some of the samples exhibiting symptoms of the disease gave an OD close to that of the healthy samples indicating low titre of virus in the samples (Bhadramurthy et al., 2005). This kind of close values to that of healthy were also reported in the detection of PYMoV infecting black pepper in Sri Lanka (de Silva et al., 2002). Other members of the badnavirus group such as SCBV, RTBV and CSSV also tend to have low absorbance values. Hence more sensitive and specific detection technique like

PCR may be necessary for samples that test negative in DAS-ELISA. However due to its cost effectiveness and adaptability in testing large number of samples, ELISA can be used for primary screening of samples (Bhadramurthy et al., 2005).

Polymerase chain reaction (PCR)

PCR (for detection of PYMoV) and reverse transcription (RT) PCR (for detection of CMV) methods are available for the detection of PYMoV and CMV infections in black pepper (Lockhart et al., 1997; de Silva et al., 2002; Siju et al., 2008; Hareesh and Bhat, 2008; Bhat et al., 2009). List of primers used for detection of PYMoV and CMV by different workers is presented in Table 4. De Silva et al (2002) and Hareesh and Bhat (2008) reported simple total DNA isolation method from black pepper which can be used as template for detection of PYMoV while Siju et al (2008) reported an efficient total RNA isolation method from black pepper that enhanced sensitivity of detection of CMV in black pepper (Fig. 7). Using PCR, Bhat et al. (2009) reported presence of PYMoV in symptomless plants of black pepper indicating the need to use sensitive methods to identify virus-free plants (Fig. 8).

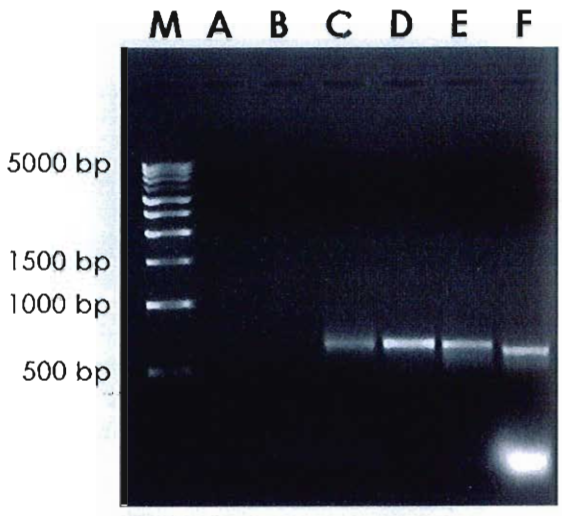

Figure 7. Detection of *Cucumber mosaic virus* in black pepper through RT-PCR. Lane M: 1 kb ladder; Lane A: Healthy control (known healthy plant); Lanes B-E: test plants Lane F: Positive control (known infected plant)

Table 4 . Oligonucleotide primers used for the detection of CMV and PYMoV infecting black pepper

Virus	Primer name	Sequence (5′ ——— 3′)	Region amplified	Amplicon size	Reference
CMV	AIB 1	ATGGACAAATCTGAATCAAC	Coat protein gene	650	Bhat et al., 2005a; Bhat and Siju 2007; Siju et al., 2008
	AIB 2	TCAAACTGGGAGCACCC			
PYMoV	AIB 35	TAACAGGACTAGGGATCG	Portion of ORF I	450	Hareesh and Bhat, 2008; Bhat and Siju 2007; Bhat et al., 2009
	AIB 36	CAGCTGGTCTTGATAATAG			
PYMoV	SCBV-R1	CTCCTTCATCTCCTCAAGAAGCCT	Portion of ORF I	700	De Silva et al, 2002
	Badna 1R	CCAAAGCTCTGATAGCAGAC			
PYMoV	AIB 104	CTATATGAATGGCTAGTGATG	Portion of ORF III	450	Bhat et al., 2009
	AIB 105	TTCCTAGGTTTGGTATGTATG			

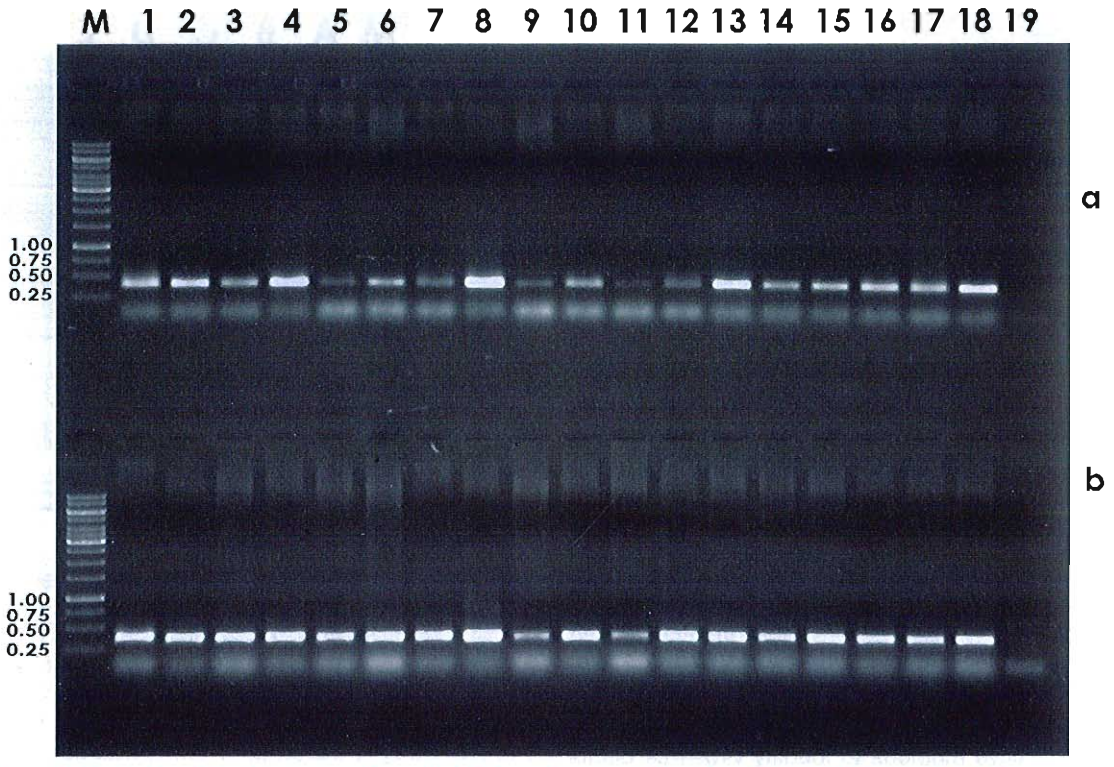

Figure 8. Indexing black pepper plants for *Piper yellow mottle virus* (PYMoV) through PCR. Lane M: 1 Kb ladder; Lane 1: Positive control (known infected plant); Lanes 2-18: test plants of var. Panchami; Lane 19: Negative control (known healthy black pepper plant) (a) PCR performed with 1.0 µl template; (b) PCR performed with 0.5 µl template

Multiplex RT-PCR

A single tube multiplex RT-PCR was developed for the simultaneous detection of CMV and PYMoV in black pepper (Fig. 9) (Bhat and Siju, 2007). The method involved combined isolation of both RNA and DNA from infected black pepper samples to use as template in mRT-PCR reaction. PCR primers designed for amplifying 650 bp from the coat protein gene of CMV and 450 bp from the open reading frame (ORF) I of PYMoV were used in the reaction. Quantity of the template used in mRT-PCR was found to be crucial for successful amplification. The method was 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 large number of plants for both the viruses.

Figure 9. 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.

Real time PCR

A SYBR Green based real time PCR for detection of PYMoV and CMV infecting black pepper was developed. The method involved isolation of template nucleic acid (both DNA and RNA) from infected plants using procedure described in Bhat and Siju (2007). The primers targeted to the conserved region in coat protein (for CMV) and ORF III (for PYMoV)

were used in the assays. The specificity of the SYBR Green assay was confirmed using virus infected sample, and healthy control. Results of real time PCR showed strong fluorescent signals (Ct values ranging from 10 - 22) only from reactions with infected samples, while the signals from healthy sample superposed to the baseline under optimized reaction conditions (Fig. 10). Specificity of the real time PCR reaction was confirmed through melt curve analysis and also by agarose gel electrophoresis. The method was validated using large number field samples of black pepper collected from different regions and other *Piper* spp. such as *P. betle, P. longum* and *P. colubrinum* (Bhat and Siljo, unpublished).

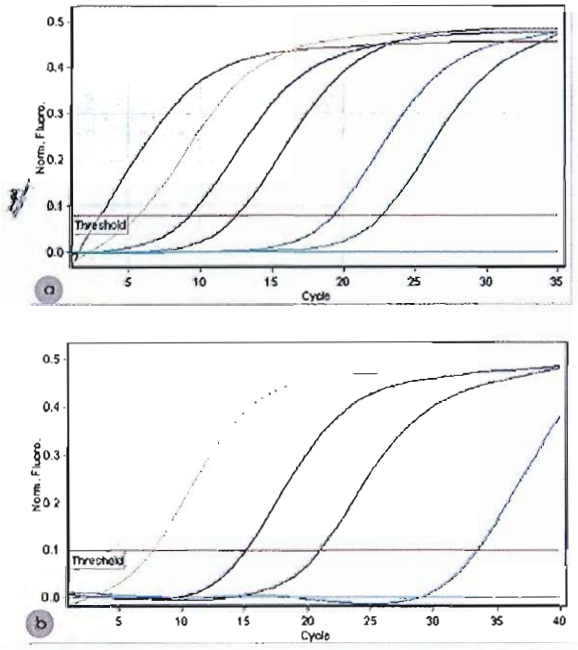

Figure 10. Real time PCR based detection of viruses in black pepper (a) CMV (b) PYMoV. Ampliification plots show the peaks and Ct values for different test samples.

Loop mediated isothermal amplification (LAMP)

LAMP is a rapid, sensitive, low cost diagnostic method that can be used for routine detection of pathogens including viruses. A rapid and sensitive detection assay based LAMP was developed for detecting PYMoV and reverse transcriptase (RT) LAMP for detection of CMV infecting black pepper. Each LAMP assay used a set of five primers designed against conserved sequences located in the viral genome. Total nucleic acids containing both RNA and DNA isolated from black pepper was used as template for both the assay. The results of LAMP reaction was assessed visually by checking turbidity, pellet formation and green fluorescence in the reaction tube and also by gel electrophoresis. The assay successfully detected both the viruses in infected plants while no cross-reactions seen with healthy plants (Fig. 11). The optimum concentrations of magnesium sulphate and betaine required for successful amplification was determined. The optimum temperature and its duration required were also determined. Sensitivity of detection limits for both the viruses by LAMP was determined using serial dilutions of total nucleic acid extracts and compared with conventional PCR and SYBR Green based real time PCR. Result showed that detection limit of LAMP was up to 100 times higher than conventional PCR and up to 10 times less sensitive than real time PCR us-

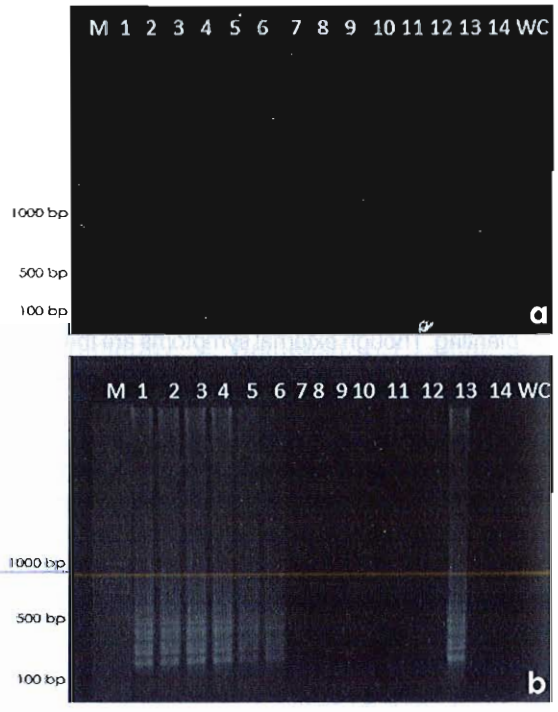

Figure 11. LAMP (A) and RT-LAMP (B) for the detection of *Piper yellow mottle virus* (PYMoV) and *Cucumber mosaic virus* (CMV) in field samples of black pepper and other *Piper* spp collected from different regions. (a) LAMP. Lane M, molecular size markers, Lanes 1 to 14 loaded with LAMP products obtained with different test samples; WC, water control. (b) RT-LAMP of the same samples as above.

ing SYBR Green. The optimized LAMP and RT-LAMP were validated by testing field samples of black pepper collected from different regions (Bhat et al., unpublished).

MANAGEMENT

Vertical transmission of the disease through planting material appears to be the major mode of disease spread since black pepper is vegetatively propagated. When infected plants are used as source of planting material, the cuttings will also be infected resulting in disease spread. Adequate care should be taken to plant healthy, virus-free cuttings, especially in new areas where the incidence of the disease is not observed in the field. Hence the primary aim should focus on production of virus-free planting materials. Insect vectors and a number of weeds and other hosts, which might act as reservoirs for the virus may also contribute to disease spread. Though use of resistant varieties is the best option to manage the disease, so far no resistant variety against the virus is available in any of the black pepper growing countries. The following measures may be adopted for controlling the disease.

PRODUCTION OF VIRUS-FREE PLANTING MATERIAL

The most successful method to control the disease would be identification and use of virus-free cuttings for planting. Though external symptoms are the good criteria for identification of virus-free plants, many of the virus infected plants remain symptomless during certain seasons. Hence there is a need to use sensitive techniques such as ELISA and PCR to identify virus-free for vegetative multiplication. Stem cuttings from indexed virus-free plants may be used for establishment of mother garden and production of nursery plants as indicated below (Bhat et al., 2010).

Establishment of mother garden

- Good bearing and disease free vines of known variety should be indexed for viruses
- Only cuttings from virus-free plants should be planted in mother garden
- It is advisable to maintain these mother plants under insect-proof conditions
- They should be periodically (at least once in a year) indexed for viruses and other pathogens
- Regular monitoring and rouging of diseased plants should be done whenever noticed
- Whenever insects (aphids, mealybugs) are seen, spraying with insecticides is necessary

Multiplication of pathogen-free planting material in nurseries

- Cuttings obtained from bearing pathogen-free mother vines are raised on a large scale in a nursery under insect-proof conditions (Fig. 12).
- The potting mixture is heat sterilized using steam or by soil solarization. Then the mixture is fortified with beneficial micro organisms such as *Trichoderma harzianum* / *Pseudomonas fluorescens*.
- Nursery plants also have to be checked for pathogens periodically (depending on the batch size of plants produced)

Batch size	Number of plants to be sampled
Up to 1000 Nos	1% plants subject to a minimum of 10 Nos
1001 to 10000 Nos	0.5% of plants subject to a minimum of 10 Nos
10001 to 100000 Nos	0.1% of plants subject to a minimum of 50 Nos

- Regular monitoring and rouging of diseased plants should be done whenever noticed
- Whenever insects (aphids, mealybugs) are seen, spraying with insecticides is necessary
- The pathogen-free stocks from the nurseries are then multiplied in secondary nurseries or used for commercial planting.

Rouging and eradication of infected plants in the field

Regular inspection and removal of severely infected plants and replanting with healthy plants should be resorted to in the field. In case of mild and moderately infected vines, additional foliar spray with micronutrient (0.25%) after spike emergence during June and spike setting during September may be undertaken. Potential weed hosts (*Amaranthus viridis, Ageratum conyzoides, Colocasia esculanta, Datura spp, Euphorbia geniculata, Oxalis corymbosa, Leucas aspera, Sonchus oleraceus* and *Synedrella nodiflora*) and crop hosts (banana, cucumbers, gladiolus, melons, tomato, brinjal, capsicum) which might act as reservoir for the virus need to be

Figure 12. Production of virus-free planting materials of black pepper. (a) Insect-proof shed used for multiplication of virus indexed black pepper (b) Multiplication of black pepper through serpentine method in an insect-proof shed.

removed. The removed plants may be burnt or buried deep in soil.

Control of vectors in the main field

Insect vectors such as aphids and mealybugs on the plant or standards once noticed should be controlled with insecticide spray. Insecticides like dimethoate 0.05% can control aphids, mealybugs and other sucking insects. Because of the closed placing of seedlings in the nursery, chances of spread of vectors are more in the nursery. Hence regular monitoring of the nursery for potential vectors is important.

FUTURE STRATEGIES

Diseases caused by viruses impose serious limitations on cultivation of black pepper in all producing countries. The key factors in any efficient disease management program are reliable identification of pathogens and understanding their natural dissemination mechanism. ELISA and PCR based techniques occupy the leading positions as methods of diagnostics and both these methods have been developed for the viruses infecting black pepper. Parameters for production of virus-free planting materials of black pepper have been developed. If used properly, this would lead to the production of disease-free certified planting material. There is a need to establish planting material production chain involving selection of parent material, initial testing and periodic testing of sub samples during multiplication and at the time of distribution of planting material. There is also a need for awareness creation on the importance of disease free planting material and capacity building of all stake holders. The future thrusts should include: (i) Development of microarray for detection of all pathogens infecting black pepper (ii) Development of easy to use diagnostic kits such as lateral flow device (iii) Development of certification programme to produce disease-free planting materials and (iv) Development of resistant varieties against viruses.

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