Journal of Tropical Agriculture 61(1): 111-116, 2023

Distribution of Stunted Disease of Black Pepper in Kerala: Varietal Response and Sero-molecular Characterization of PYMoV

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Received 29 August 2022; received in revision form 31 March 2023; accepted on 29 May 2023

Abstract
Study on viruses infecting black pepper indicated that infection of Cucumber mosaic virus (CMV) and Piper yellow mottle virus (PYMoV) either alone or as mixed infections was prevalent in cultivated areas of Idukki and Wayanad districts of Kerala. Highest disease incidence (DI) was observed in Poothadi (45.50 %) of Wayanad and lowest in Karunapuram (21.62 %) of Idukki district. Highest vulnerability index (V.I.) was recorded in Panamaram (61) of Wayanad and lowest in Pampadumpara (32) of Idukki. Serological technique DAS-ELISA was used for detection of these viruses along with molecular detection using coat protein specific primers. The isolates of PYMoV from different locations ofIdukki and Wayanad showed similarity in ORF III to other reported PYMoV sequences.

Key words: *Piper yellow mottle virus* (PYMoV), *Cucumber mosaic virus*(CMV), Black pepper, ELISA, PCR

Introduction

Black pepper known as "King of Spices" and "Black Gold" is one of the most valuable and earliest known spices in the world. Black pepper (*Piper nigrum* L.), which originated in the tropical evergreen forests of the Western Ghats in India has captured global attention and ardor that widened over the years. Viral diseases are the third among the serious diseases of black pepper after foot rot and anthracnose. Since black pepper is a vegetatively propagated crop, chances of spreading virus among plants is high. Sarma et al.(2001) reported the occurrence of *Cucumber mosaic virus* (CMV) infecting black pepper in India. Bhat et al. (2005a) reported the presence of a badna virus (*Piper yellow mottle virus*) associated with black pepper which is transmitted by mealy bug. The present study was undertaken with the objectives like survey and collection of viruses infected black pepper plants from Idukki and Wayanad districts, symptomatology studies, varietal response, serological diagnosis and molecular characterization of the viruses.

Materials and methods

Survey and collection of virus infected black pepper plants

Survey was conducted in the major pepper growing tracts of Kerala, Wayanad and Idukki. Erattiyar, Kattappana, Pampadumpara and Karunapuram panchayats were selected from Idukki district. From Wayanad district Ambalavayal, Meenangadi, Poothadi and Panamaram were surveyed. Fields of five farmers were taken for survey from each panchayat. From the farmer's field, disease

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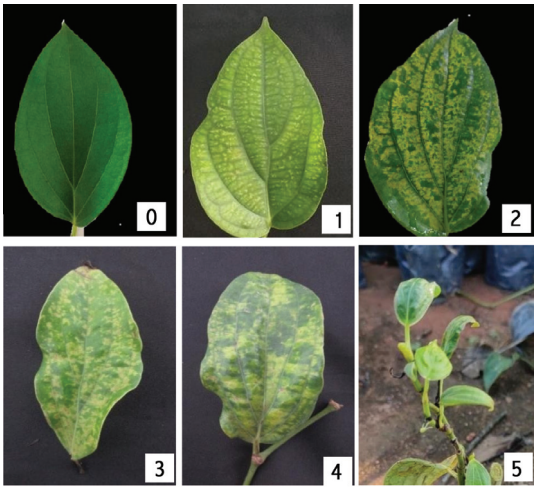

0- No symptom, 1- Very light mottling of younger leaves, 2-Mottling of leaves and vein clearing 3- Leafc urling and distortion of leafsize, 4- Distortion and reduction of leaf size, 5-Stunting of the plant

Plate1. Diseases core chart ranging from 0-5

incidence and severity were recorded. Pathometry was done using a disease score scale of 0-5 (Plate 1) and calculation of vulnerability index was done as per the equation (Bos, 1982).

$$V.I. = \frac{(0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4 + 5n_5) \times 100}{n_t(n_c-1)}$$

$n_0, n_1 \ldots n_5$ = Number of plants in category 0, 1, 2, 3, 4 and 5

n_t = total number of plants

n_c = total number of categories

Response of 15 black pepper varieties (KAU, IISR and local) was undertaken by wedge grafting the infected scions showing the symptoms of both viruses (CMV and PYMoV) on to healthy black pepper plants.

Serological and molecular characterization of viruses

Samples were subjected to DAS-ELISA for serological detection and DNA isolation was done using CTAB method (Doyle and Doyle, 1990).

Amplification and Sequencing

The isolated DNA was subjected to PCR for amplification. Primers specific to ORF III of PYMoV was used. Forward primer 5' CTATATGAATGGCTAGTGATG 3' and reverse primer

5'TTCCTAGGTTTGGTATGTATG3' were used (Bhat and Siju, 2009). The positive reaction was considered as amplification at 400 bp. Amplified product was sequenced at Agrigenome Pvt. Ltd., Kochi.

Blast analysis and construction of phylogeny tree

Sequencing was done for the amplified PCR products and subjected to BLAST analysis to study sequence similarity of the isolated samples with already reported sequences at the National Centre for Biotechnological Information (NCBI) site. Sequences were aligned in MEGA 6.0 software to construct the phylogeny tree.

Results and discussion

The survey revealed that viral diseases of black pepper are wide spread in these regions. Disease incidence varied from 21- 37 per cent in Idukki district while it varied between 24- 45 per cent in Wayanad district. Highest disease incidence was noted in Erattiyar panchayat (37.40 %) and lowest in Karunapuram panchayat (21.62 %) whereas in Wayanad district highest incidence was recorded at Poothadi (45.50 %) and lowest in Meenangadi panchayat (24.50 %). Highest vulnerability index was recorded in Erattiyar panchayat (45) of Idukki

Table1. Disease incidence and vulnerability index observed for different black pepper samples collected during survey

Sl.No.	Sample name	DI (%)	VI
1	Erattiyar	37.40	45.00±0.577
2	Kattappana	32.40	41.00±0.577
3	Pampadumpara	29.72	32.60±0.231
4	Karunapuram	21.62	41.00±1.764
5	Ambalavayal	42.20	37.00±2.028
6	Meenangadi	24.50	38.20±0.306
7	Poothadi	45.50	58.00±0.577
8	Panamaram	36.41	61.00±2.309

district while lowest was recorded at Pampadumpara (32). In Wayanad highest VI was recorded in Panamaram (61) and lowest in Ambalavayal (37) (Table 1). Bhat et al. (2005b) conducted studies regarding the stunted disease incidence in Karnataka and Kerala. Highest disease incidence was found in Wayanad (45.4 per cent) and Idukki (29.4 per cent) districts of Kerala. Studies conducted at Kodagu district of Karnataka recorded a disease incidence of 14.9 per cent and 5.2 per cent disease incidence in Hassan district (Bhat et al., 2005b). The results of the survey confirmed the observations.

Diseased samples were collected from the surveyed locations to study the symptomatology of virus infection. Various symptoms observed on the field were chlorotic spots, vein banding, distortion and reduction in leaf size, general chlorosis, puckering of leaves, and vein clearing. Symptoms initially appear as small chlorotic spots and gradually lead to inter venal chlorosis, vein clearing, severe mottling, and vein banding. Reduction in leaf size, distortion in shape of leaf, and stunting of growth with slight mottling were also observed in virus infected plants. Karimunda was identified as the most susceptible variety with highest V.I. (48) followed by Panniyur 6, Sakthi, Thevam, and Girimunda.

Serological technique *viz*., double antibody sandwich ELISA (DAS-ELISA) was done using polyclonal antibodies of Cucumber Mosaic Virus (CMV) and two monoclonal antibodies of Banana Streak Virus (BSV). Since the availability of PYMoV antisera is meager; it was imperative to try for other related antisera *viz*, BSV and SCBV. From both Idukki and Wayanad 20 samples with three replications were collected and analyzed. Primary antibody of CMV was used at a dilution of 1:1000 and that of BSV was used at 1: 200. Secondary antibodies were used at a dilution of 1: 10,000. DAS ELISA was found to be sensitive in detecting the presence of CMV and PYMoV from the diseased samples. Infection of PYMoV was found to be more predominant than CMV. Out of 40 samples tested 10 samples were infected with CMV, 21 samples were infected with PYMoV and mixed infection was found in 4 samples. It was found that mottling along with curling is associated with PYMoV infection; reduction and distortions in leaf size were found associated with CMV infection which was confirmed using serological tests.

Association of a Cucumber mosaic virus was reported with black pepper in many pepper growing areas. Bhat et al. (2003) reported disease occurrence as either due to pure infection by CMV or PYMoV or due to mixed infection by both the viruses. The intensity of disease was found more in case of mixed infections. When mixed infections lead to increased severity of the disease it is referred to as a synergistic disease. A few of the synergistic diseases reported are corn lethal necrosis in corn (Niblett et al., 1978), and cowpea stunt (Pio-Reibero et al., 1978). In the present study, mixed infection was observed in Panamaram panchayat of Wayanad district. The high disease severity observed for Panamaram can be substantiated due to the detection of both viruses on the serological diagnosis.

Selvarajan et al. (2009) studied the mitigation of banana bract mosaic disease and compensation of

Table2. Response of varieties to graft transmission of viruses

Sl. no	Variety	Days taken for	Nature of symptom symptom expression	Vulnerability index	Inference
1	Karimunda	40	Chloroticspots	48	S
2	Panniyur6	55	Chloroticspots	42	MS
3	Thevam	58	Chloroticspots	32	MS
4	Shakti	60	Chloroticspots	28	MS
5	Girimunda	75	Chloroticspots	28	MS

S-Susceptible, MS-Moderately susceptible

yield loss through a higher dose of fertilizer application. Umadevi et al. (2016) reported severe symptom expression in plants growing under poor soil nutrient status and temperature stress. In the present survey, increased disease incidence was found in poorly managed fields than in well maintained fields which is in concurrence with the above mentioned results.

Sarma et al. (2001) observed symptoms like interveinal yellow flecking, yellow mottling, dark-green vein banding, narrow distorted leathery leaves, and an overall reduction in growth associated with CMV infection in black pepper. Stunting, reduction, and distortion of leaf size were found associated with samples collected from Kattappana, Poothadi, and Panamaram panchayats where the presence of CMV was identified based on serology. Stunting was less frequently observed, while reduction and distortion of leaf size were found in samples collected from the above listed panchayats. In some samples, an admixture of all the described symptoms was observed indicating mixed infection of viruses. Hence symptomatology alone cannot be used for the successful detection of viruses emphasizing the need for serological and molecular detection techniques.

In the present study, some samples expressing symptoms were found to have low absorbance value which might have been due to lower value in titration. The genomic and serological heterogeneity of badna viruses can be a reason for the lesser sensitivity of serological methods. The varying OD values found in the positively tested samples indicate the varied concentration of virus at different locations. Comparison of symptoms with the serological results obtained in ELISA showed that mottling with curling is associated with infection of PYMoV whereas reduction and distortion in leaf size were observed with CMV infection. Ayisha (2010) observed that leaves showing general chlorosis reacted positively to both CMV and PYMoV while sickle shaped leaves were found to be infected with CMV. Leaf samples with severe

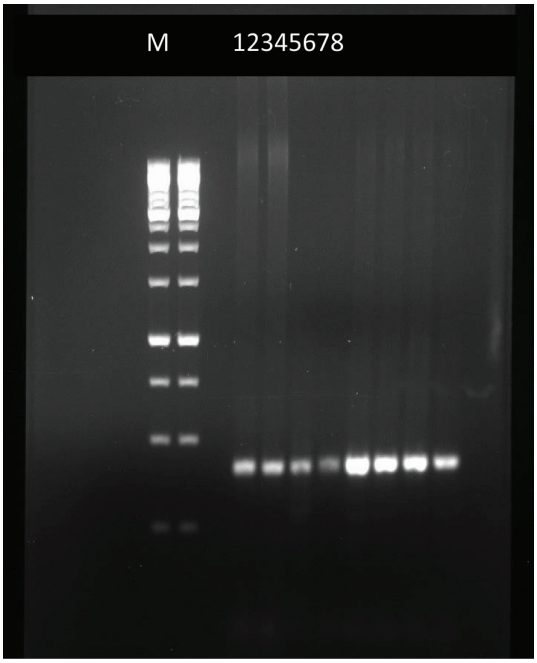

Plate2. Electrophoresis gel image of amplified DNA of PYMoV infected samples from Idukki and Wayanad, lanes M- 1000 bp DNA ladder; lane 1-8: isolates from Erattiyar, Kattappana, Pampadumpara, Karunapuram, Ambalavayal, Meenangadi, Poothadi and Panamaram panchayats.

chlorosis and mottling were found to react to SCBV, which is serologically related to PYMoV.

PCR amplification of ORF III was done using specific primers and agarose gel electrophoresis was conducted. Amplicons of size 400bp were obtained when compared with a 1 kb DNA ladder (Plate 2). Bhat et al. (2009) conducted PCR detection based on the ORF III sequence of the PYMoV infecting black pepper in India (Gen Bank accession No. DQ836227) and for a positive reaction, bands of 400 bp were obtained. All the isolated sequences were found to be related to PYMoV. Phylogenetic studies revealed that isolates from Idukki and Wayanad were grouped in separate clades. Isolates from Erattiyar, Kattappana, and Pampadumpara were clustered together indicating that they are closely related while isolates from Ambalavayal, Meenangadi, Poothadi, and Panamaram formed a

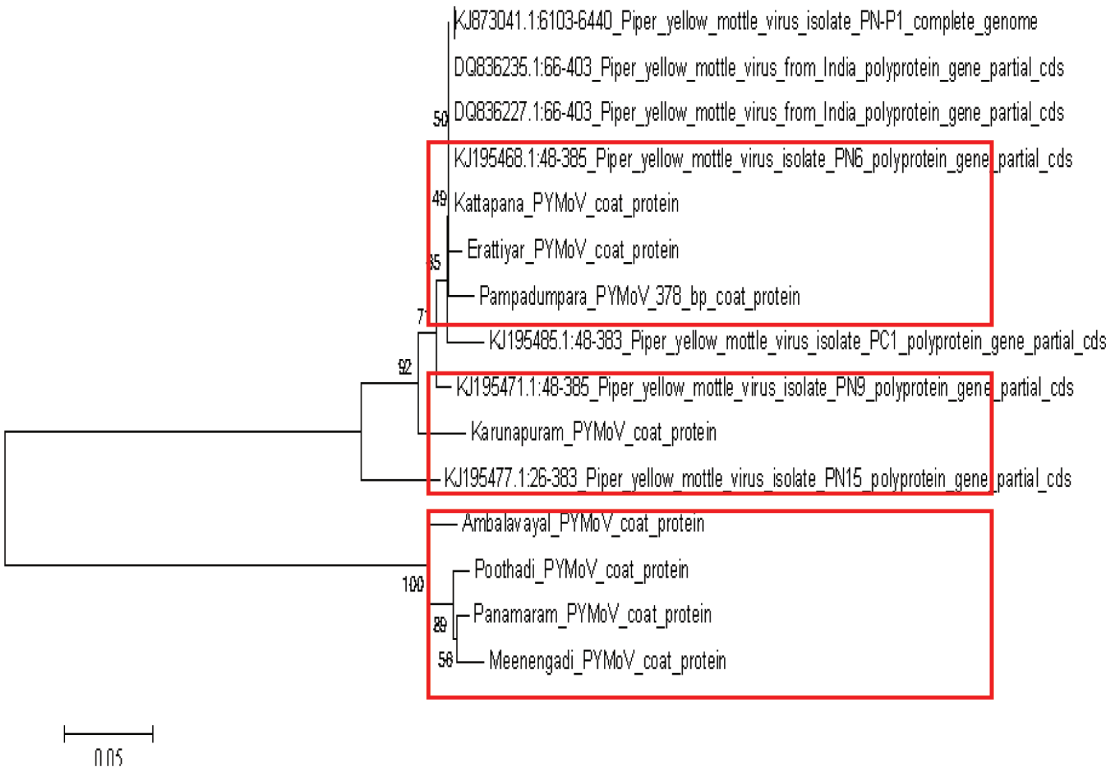

Plate3.Phylogeny tree showing the virus isolates in three different clades

separate clade (Plate3).

Badna viruses are known to infect many crops like banana, and cocoa which are found in a mixed cropping system along with black pepper. Hence the similarity of PYMoV to other badna virus needs to be studied further to understand whether this virus can act as an infection source for black pepper.

Acknowledgement

This work was supported by Kerala Agricultural University.

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