VirusDis. (April–June 2021) 32(2):305–313
https://doi.org/10.1007/s13337-021-00686-3

ORIGINAL ARTICLE

Symptoms of piper yellow mottle virus in black pepper as influenced by temperature and relative humidity

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Received: 5 February 2021 / Accepted: 26 March 2021 / Published online: 12 April 2021

Abstract Masking of symptoms in winter and their re-appearance in black pepper (*Piper nigrum* L.) infected with piper yellow mottle virus (PYMoV) in summer is common, especially on new flushes that appear after pre-monsoon showers. Plants of nineteen cultivars of black pepper infected with PYMoV but without any visible symptoms were grown in a polyhouse under natural conditions and in a greenhouse under controlled conditions from January 2019 to January 2020. The number of plants expressing symptoms in the polyhouse increased gradually from 1% during the 3rd standard meteorological week (SMW) (16 January) to 41% during the 21st SMW (22 May), when the afternoon temperature was 30–40 °C and relative humidity (RH) was 75–93%, but began declining thereafter until the 53rd SMW (1 January), when the afternoon temperature was 30–36 °C and RH was 65–86%. The proportion of plants expressing symptoms varied with the cultivar. However, in the greenhouse, in which temperature and RH were maintained at approximately 26 °C and 80%, respectively, not more than 2% of the plants expressed symptoms. The number of symptomatic plants was positively correlated to maximum temperature (T Max) and maximum relative humidity (RH Max) in the afternoon. Based on this observation, a model for predicting the percentage of symptomatic plants was developed using stepwise regression analysis. Plants at the two sites did not differ significantly in the concentration of virus (virus titre) but differed significantly in the content of total carbohydrates, lipid peroxidase, and phenols.

Keywords Biochemical assays · Real-time reverse transcription-polymerase chain reaction · Standard meteorological week (SMW) · Virus titre

Introduction

Temperature plays a crucial role in host–pathogen interactions at multiple levels including the transcriptome, cellular, and physiological levels [1, 35] and may influence the course of infection by regulating viral replication, host metabolism, and infectivity. Temperature-driven modifications in host physiology may alter virus–host interactions by making the host either resistant or susceptible to the virus [12]. Virus replication and virus–host interaction within the host are also influenced by the season. Virus build-up in freshly formed leaves of *Arabidopsis halleri* infected with turnip mosaic virus was temperature-dependent, its effect being evident in winter [18]. Temperature-sensitive resistance to plant viruses has been reported in several host–virus systems including cassava and cassava mosaic geminiviruses [7], tobacco and tobacco mosaic virus [19], *Nicotiana* spp. and tobacco ringspot virus [32], and wheat and wheat streak mosaic virus [42]. In most of these cases, attenuation of symptoms due to temperature changes in a host is attributed to weaker RNA-silencing activity or to temperature-dependent expression of host microRNAs and target mRNAs [37, 40]. Higher temperatures enhanced the replication of turnip crinkle virus (TCV) [44] and enabled tobacco mosaic virus and TCV to spread by weakening the plant's defences [19]. In some cases, the

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13337-021-00686-3.

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expression of symptoms in virus-infected plants was muted in plants grown at higher temperatures [7, 38, 40]. The severity of diseases caused by begomoviruses was higher under wet and humid conditions than that under dry conditions [28]. A few endogenous pararetroviruses such as petunia vein clearing virus, banana streak virus (BSV), and tobacco vein clearing virus are activated when plants are subjected to abiotic stress including that from unsuitable temperatures [23, 30, 43]. Viruses are also known to alter photosynthesis and carbohydrate partitioning in infected leaves [15, 16, 41].

Black pepper (*Piper nigrum* L.) is native to southern parts of the mountain range known as the Western Ghats because it flanks India's west coast. The crop is grown mainly in Kerala and Karnataka, two of the country's southern states. Diseases, particularly virus diseases, are a major threat to pepper because viruses are systemic and spread through vegetative propagation, the most common method of multiplying black pepper. Piper yellow mottle virus (PYMoV), a serious threat to black pepper production worldwide [4, 22], is a member of the genus *Badnavirus*. The virus is bacilliform-shaped and contains circular covalently closed double-stranded DNA genome of about 7.5 kb encapsidated in non-enveloped bacilliform virions. Plants infected with PYMoV are characterized by chlorotic, mottled, vein-cleared, and distorted leaves and stunted growth. Symptom expression in many badnaviruses is known to be temperature dependent [9, 11]. Although asymptomatic initially, infected plants showed more severe symptoms when exposed to abiotic stress, such as that triggered by changes in temperature or by nutrient deficiency [4]. In our earlier study, asymptomatic PYMoV-infected plants of black pepper 'Panniyur-1′ developed symptoms within 10 days of being exposed to a temperature of 35 °C [39] the present investigation extends that study to nineteen cultivars of black pepper and to the effects of relative humidity (RH) as well.

Materials and methods

Source of plants and detection of the virus

In two sets of asymptomatic rooted cuttings of PYMoV-infected plants, the infection was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) assay. The following nineteen cultivars were chosen for the study, all of them popular in Kerala and Karnataka: Arka Coorg Excel, IISR-Girimunda, IISR-Malabar Excel, IISR-Shakthi, Panchami, Panniyur-1 to Panniyur-9, PLD-2, Pournami, Sreekara, Subhakara, and Vijay. All the rooted cuttings were obtained from the nursery of the ICAR-Indian Institute of Spices Research (ICAR-IISR), Kozhikode, Kerala, India.

For detecting PYMoV in the plants, total RNA was isolated as described earlier [33] from young leaves (the first fully expanded leaf from the tip of each plant), 50 mg each, and RNA samples were dissolved in 50 µL of sterile water; from this solution, 1 µL (about 100 ng) was used as the template for RT-PCR, which was carried out using the total RNA as the template, 5′-GAGTACCAACAG GTGATGA-3′ as the forward primer, and 5′-GTGCT TCCTCTTCTCAATC-3′ as the reverse primer corresponding to the open reading frame 3 of PYMoV as described previously [2]. The reaction mix consisted of 1 µL of total RNA, 1 × *Taq* polymerase buffer, 1.5 mM $MgCl_2$, 400 µM dNTP mix, 10 mM dithiothreitol, 10 pM each of forward and reverse primers, 1 U of RNase inhibitor, 1.25 U of RevertAid reverse transcriptase, 0.75 U of *Taq* polymerase, and enough RNase-free water to make up the volume to 50 µL. The temperature profile in the thermal cycler involved cDNA synthesis at 42 °C for 45 min followed by 35 cycles at 94 °C for 30 s, at 50 °C for 40 s, and at 72 °C for 1 min, with the final extension at 72 °C for 10 min. The products of RT-PCR were run on 1% agarose gel electrophoresis for 1 h at 100 V and checked for the presence of the band of the expected size (539 bp) using a GelDoc system. One set of the RT-PCR positive plants (30 plants of each cultivar) was raised in a polyhouse, under natural conditions, and the other (10 plants of each cultivar) in a greenhouse under controlled conditions.

Exposure to temperature stress and expression of symptoms

The experiments were conducted, and the observations recorded, during standard meteorological weeks (SMW) from January 2019 to January 2020. Temperature and RH were recorded in the polyhouse daily in the morning (8.30 a.m.) and in the afternoon (2.30 p.m.); in the greenhouse, the temperature was maintained at approximately 26 °C and RH, at 80%. The number of plants expressing symptoms was recorded for each cultivar in the polyhouse and in the greenhouse on the 7th day of every SMW. The disease incidence (DI) for each cultivar in each SMW was determined as follows: DI = number of plants of a given cultivar showing symptoms/total number of plants of that cultivar × 100). The average values of RH and temperature in every SMW were calculated and plotted against the DI. The data were further subjected to correlation and regression analysis to determine the relationship of the DI to temperature and to RH using IBM SPSS ver. 25.

Determination of PYMoV titre in plants

PYMoV titre in 3 plants from each variety chosen at random was determined using SYBR-Green RT-PCR once in three months using the protocol described earler [6]. Total RNA isolated from leaves (the first fully expanded leaf from the tip of each plant) as described above was dissolved in 50 µL of water. Real-time RT-PCR reaction was carried out in triplicate. The mix contained 12.5 µL of 2 × QuantiFAST SYBR Green PCR Master Mix (Qiagen, Germantown, Maryland, USA), 10 µM each of the two primers (forward primer 5′-CACTTAGTCGCAATGCT GGA-3′ and reverse primer 5′-CCAATAGTTGCTCCC AGGAA-3′ corresponding to the open reading frame 3 of PYMoV), 25 U of Revertaid reverse transcriptase (Fermentas, Vilnius, Lithuania) and 1 µL of template RNA (approximately 100 ng). The thermal cycler (Rotor-Gene, Qiagen, Hilden, Germany) was programmed for cDNA synthesis at 42 °C for 45 min and initial denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 15 s and 60 °C for 20 s. The cycle threshold (Ct) values were recorded and subjected to the *t*-test to find out if they were significant. Following real-time RT-PCR, the amplicons were subjected to melt analysis from 60 to 95 °C to check the specificity of the product.

Biochemical assays

One variety each representing high, medium, or low DI was selected from the polyhouse as well as the greenhouse for biochemical assays. Young leaves were collected from the representative plants in the 21st SMW (22 May) from both sites and ground in liquid nitrogen separately using pestle and mortar. The ground samples, lyophilized and stored in the cold room, were used for the estimation of total proteins [24], phenols [20], total non-structural carbohydrates and chlorophyll [31], lipid peroxidase activity [17], and super oxidase dismutase activity [3]. All the assays were repeated three times and the results were analysed using the *t*-test.

Results

Effect of temperature and relative humidity on symptom expression

The weekly mean values of temperature, RH, and the percentage of plants expressing symptoms were recorded for each variety in the polyhouse and in the greenhouse (Tables S1). The symptoms of the PYMoV infection included mild to moderate yellow mottling followed by deformation of the leaf, reduction in the intermodal length and stunting of plants (Fig. 1). The number of plants expressing symptoms in the polyhouse varied within and among the cultivars from the 1st to the 53rd SMW from January 2019 to January 2020. Fluctuations in temperature and RH affected the number of symptomatic plants (Fig. 2). In general, the average number of symptomatic plants of all cultivars showed a gradual increase from 1% during the 3rd SMW (16 January) to 41% during the 21st SMW (22 May) as the afternoon temperature increased from 30 °C to 40 °C and RH, from 75 to 93% (Fig. 2). Thereafter, the numbers began to decline until the 53rd SMW (1 January) as the afternoon temperature dropped from 36 to 30 °C and RH, from 86 to 65%.

Among the nineteen cultivars, 83–100% of the plants of Arka Coorg Excel, Panniyur-6 and Panniyur-2 expressed symptoms during the 21st, 19th, and 16th SMW (22 May, 8 May, and 17 April), respectively, and the average DI in these three cultivars was 21–51% (Table S1; Fig. 2). In contrast, the highest percentages of symptomatic plants were recorded in Panniyur-9 (9%), IISR-Malabar Excel (7%), and Pournami (6%) during the 19th SMW (8 May) and 21st SMW (22 May), and the average DI in these varieties was only 2%. The maximum percentage of symptomatic plants in the remaining thirteen cultivars was 20–59%, recorded from the 21st SMW (22 May) to the 24th SMW (12 June), and the average DI of these cultivars was 4–20%. Based on the percentage of plants expressing symptoms, Arka Coorg Excel, Panniyur-2, and Panniyur-6 were placed into the high-symptom-expression category (DI from 0 to 100%); IISR-Malabar Excel, Panniyur-9, and Pournami, into the low-symptom-expression category (DI from 0 to 9%); and the rest, into the medium-symptom-expression category (DI from 0 to 59%). In contrast to the plants maintained in the polyhouse, only 0–2% of all the plants in the greenhouse expressed symptoms.

Disease incidence was positively correlated to the *afternoon* maximum temperature (T Max) and afternoon maximum RH (RH Max) (except in Panniyur-6 and Panchami) whereas neither *morning* temperature nor morning RH showed any significant correlation with the DI (Table 1). A model to predict the incidence of PYMoV disease in each of the three categories of the cultivars was developed based on one-year data using stepwise regression analysis (Table 2). The values predicted by the model showed a good fit with the actual values, as can be seen in the normalized plot fit (Fig. 3a–c).

Virus titre

The concentration of the virus (virus titre) in the plants showed no difference between the plants raised in the polyhouse and those raised in the greenhouse except in August (Table S2). In general, most cultivars, whether

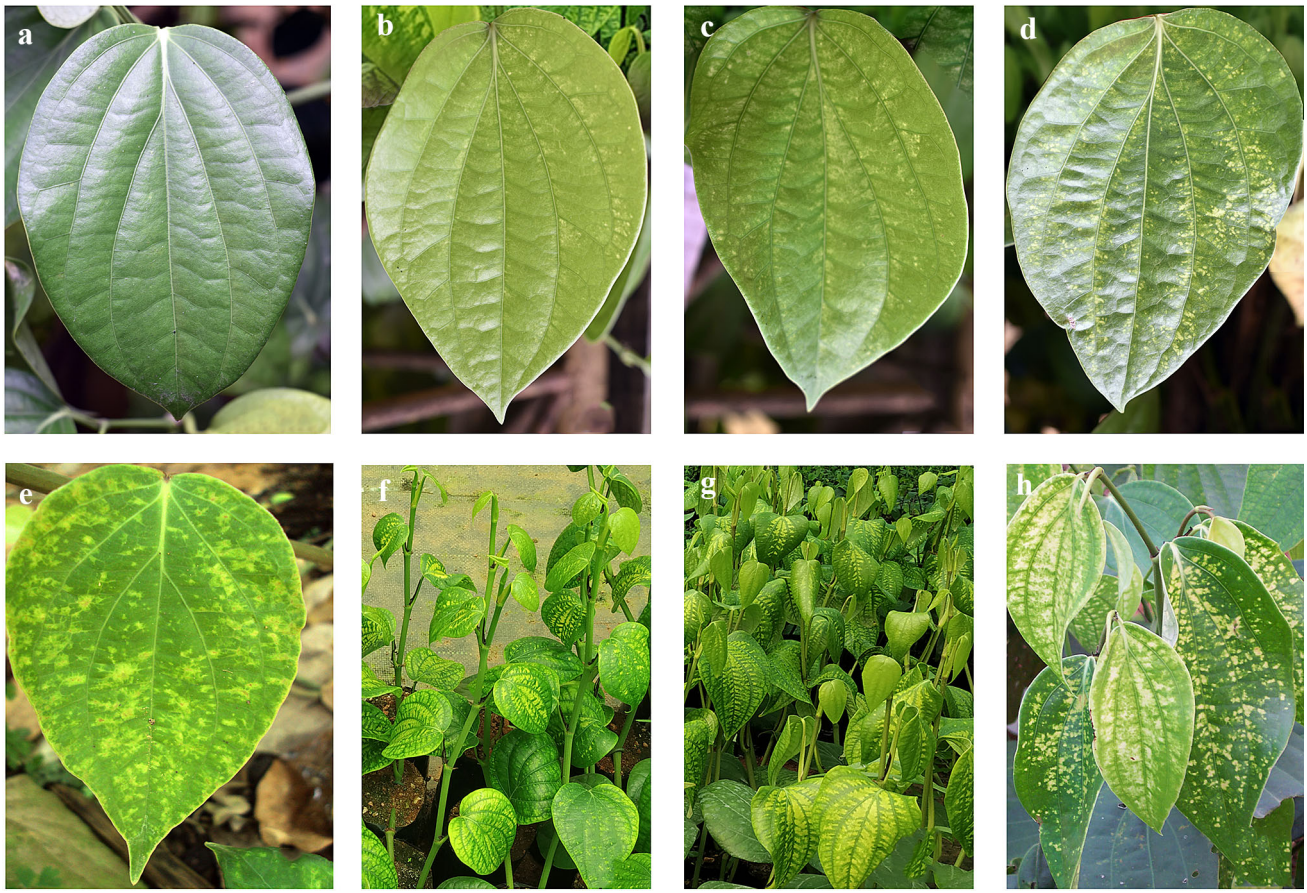

Fig. 1 Symptoms of piper yellow mottle virus on black pepper plants grown in a polyhouse (under natural conditions). **a** asymptomatic (apparently healthy) plants, **b**, **c** mild and moderate yellow mottling, **d**, **e** mottling and leaf deformation, **f**, **g** severely infected plants, **h** close-up view of a severely infected plant

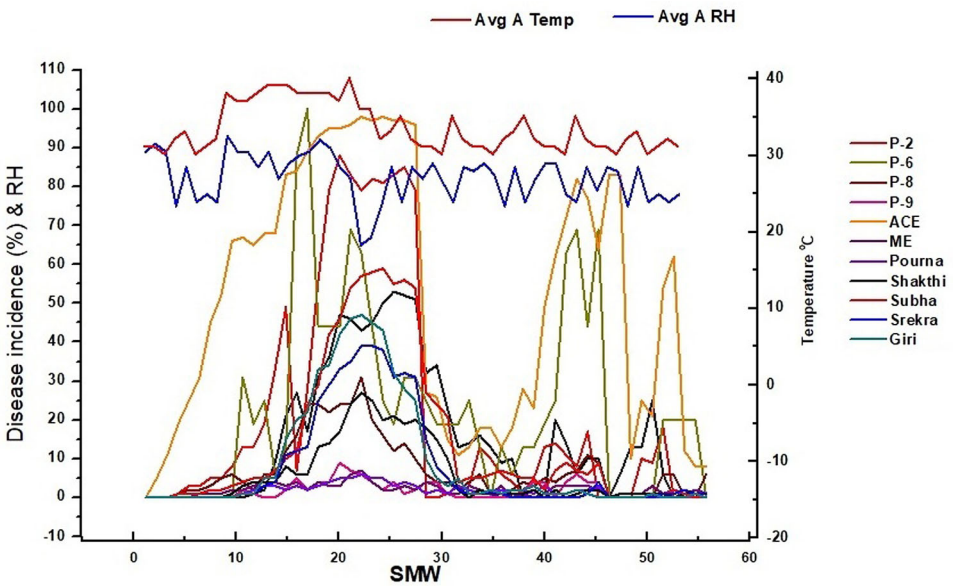

Fig. 2 Percentage of disease incidence in selected cultivars of black pepper grown in a polyhouse (under natural conditions) from the 1st to the 53rd standard meteorological week (SMW). Average afternoon temperature (Avg A Temp) and afternoon relative humidity (Avg A RH) recorded at 2.30 p.m. in each SMW in the polyhouse are also shown. ACE (Arka Coorg Excel), Giri (IISR-Girimunda), ME (IISR-Malabar Excel), Shakthi (IISR-Shakthi), P-2 to P-9 (Panniyur-2 to Panniyur-9), Pourna (Pournami), Srekra (Sreekara), Subha (Subhakara)

Table 1 Correlation analysis between temperature and relative humidity with symptom expression in piper yellow mottle virus infected black pepper plants of different varieties

Parameter	P-1	P-2	P-3	P-4	P-5	P-6	P-7	P-8	P-9
M-temp	− 0.048	0.162	0.153	0.402**	0.136	0.063	0.138	0.216	0.081
M-rh	− 0.182	− 0.283*	− 0.286*	− 0.328*	− 0.200	− 0.062	− 0.172	− 0.256	− 0.121
A-temp	0.392**	0.587**	0.600**	0.840**	0.402**	0.493**	0.571**	0.702**	0.438**
A-rh	− 0.445**	− 0.580**	− 0.418**	− 0.464**	− 0.472**	− 0.217	− 0.506**	− 0.551**	− 0.360**

Parameter	Vijay	PLD-2	ACE	Giri	ME	Pornmi	Subha	Shkti	Srekra	Panch
M-temp	0.311*	− 0.150	0.256	0.164	0.237	0.115	0.007	0.048	0.108	0.142
M-rh	− 0.192	− 0.153	− 0.220	− 0.237	− 0.202	− 0.303*	− 0.211	− 0.199	− 0.218	− 0.301*
A-temp	0.701**	0.419**	0.643**	0.617**	0.605**	0.614**	0.472**	0.488**	0.555**	0.613**
A-rh	− 0.456**	− 0.328*	− 0.366**	− 0.625**	− 0.334*	− 0.519**	− 0.546**	− 0.514**	− 0.621**	− 0.561**

M-temp (morning temperature at 8:30 a.m.); M-rh (morning relative humidity at 8:30 a.m.); A-temp (afternoon temperature at 2.30 p.m.); A-rh (afternoon relative humidity at 2.30 p.m.); P-1 to P-9 (Panniyur-1 to Panniyur-9); ACE (Arka Coorg Excel); Giri (IISR-Girimunda); ME (IISR-Malabar Excel); Pornmi (Pournami); Subha (Subhakara); Shkti (IISR-Shakthi); Srekra (Sreekara); Panch (Panchami)

*Significant at 5%; **highly significant at 1%

Table 2 Prediction model for symptom expression in piper yellow mottle virus (PYMoV) infected black pepper of different varieties

Disease incidence category	Regression equation	R^2 value
High	$Y_{DI} = -58.56 + 4.70$ (A-temp) $- 0.82$ (A-rh)	0.4983
Medium	$Y_{DI} = -2.66 + 1.78$ (A-temp) $- 0.55$ (A-rh)	0.5302
Low	$Y_{DI} = 0.25 + 0.49$ (A-temp) $- 0.17$ (A-rh)	0.4502

Where, Y_{DI} is PYMoV disease incidence (%), A-temp is afternoon maximum temperature at 2:30 pm in polyhouse (°C) and A-rh is afternoon maximum RH at 2:30 pm in polyhouse conditions. The regression values for high, medium and low PYMoV incidence varieties is 0.4983, 0.5302 and 0.4502 respectively which indicates that variability of disease incidence was 49, 53 and 45% in the model

under natural conditions (the polyhouse) or under controlled conditions (the greenhouse), showed high Ct values, from 14.5 to 25.8, indicating low virus titre during the 8th SMW (21 February) (Table S2). Similarly, most cultivars recorded low Ct values, from 9.6 to 23.6, indicating high virus titre, in the polyhouse but high Ct values, from 14.6 to 27.2, in the greenhouse during the 51st SMW (21 December). The specificity of the real-time RT-PCR product was confirmed through melt curve analysis, which showed a single peak at 81 °C, indicating that only the target fragment had been amplified.

Biochemical assays

Plants from the two sites differed significantly in terms of carbohydrate content, lipid peroxidase activity, and phenol content but not in terms of chlorophyll content, protein content, and superoxide dismutase activity (Table S3). The contents of carbohydrates, phenols, and proteins and superoxide dismutase activity were significantly higher, and chlorophyll content and lipid peroxidase activity were significantly lower, in plants in the greenhouse compared to the corresponding values in plants from the polyhouse. Phenol content was maximum in Panniyur-1 grown in the greenhouse and the activity of superoxidase dismutase was maximum in Arka Coorg Excel grown in the polyhouse.

Discussion

Yellow mottle disease caused by PYMoV is reported from all black-pepper-growing regions in the world. Masking of symptoms and their re-emergence on flushes of new growth and milder symptoms or no symptoms at all on other leaves are common under field conditions [13, 22]. Losses due to the disease in terms of yield vary from negligible to 85% [5]. Temperature and RH are the two important abiotic factors that influence disease incidence and severity. In the present experiment, the symptoms were more severe at higher temperatures and higher RH in all the cultivars—which were asymptomatic at lower temperatures and lower RH. Higher incidence and severity of yellow mottle disease in black pepper exposed to high temperatures were also reported earlier [39]. According to Lockhart [21] the

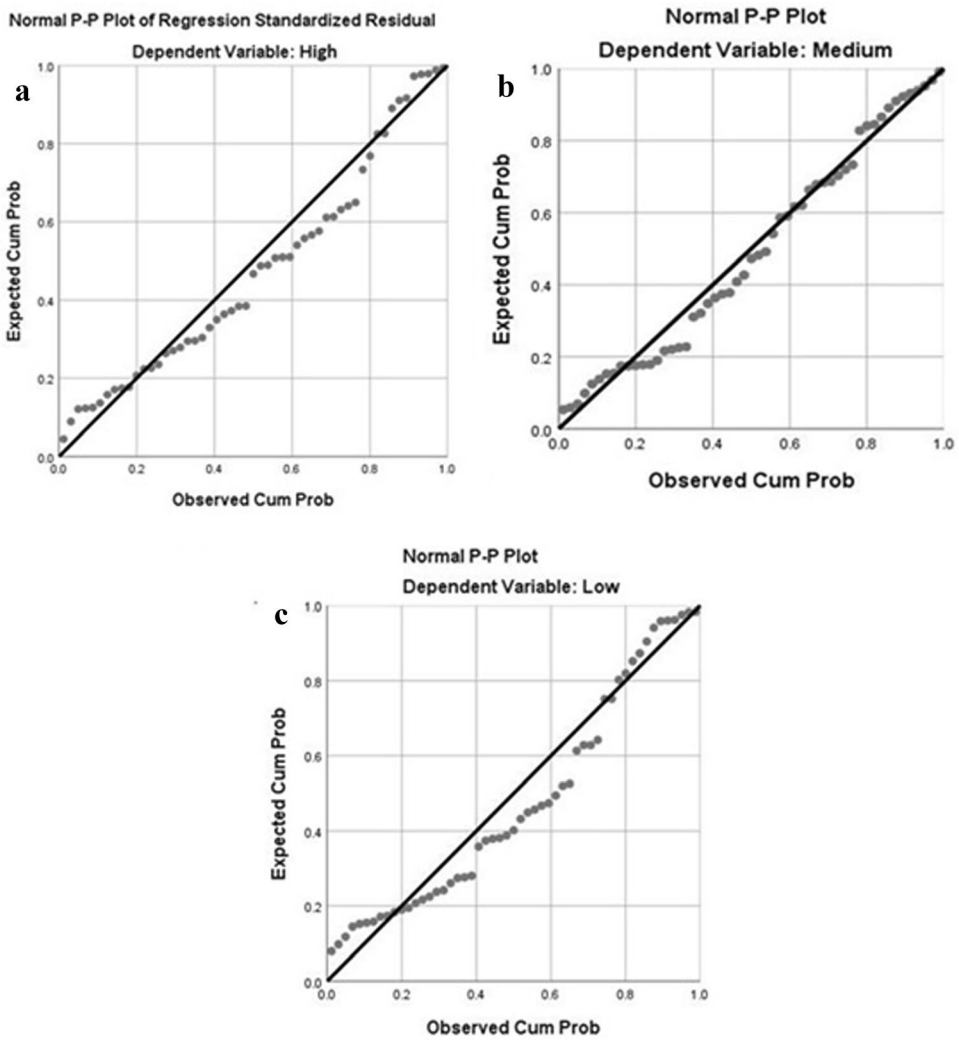

Fig. 3 Normalized plot fit model. **a** High disease incidence (DI), **b** medium DI, and **c** low DI of piper yellow mottle virus (PYMoV) in different cultivars of black pepper as predicted by the model

streaks on banana leaves due to BSV were pronounced at high temperatures, although Dahal et al. [9] reported that it was the temperature regime rather than absolute temperature that was important for the expression of severe symptoms. The incidence of BSV varied significantly with the season during a cropping cycle, being higher during the rainy season (28–30 °C) and low or negligible during the hot, dry season (28–35 °C) [10]. The incidence of BSV was maximum in the wet season but the incidence could not be linked to low temperatures [11]. Temperature-dependent susceptibility was reported in potato in the case of some strains of potato virus Y (PVY). Potato cultivar 'Rywal' showed a hypersensitive reaction (HR) to both PVY^{O} and PVY^{N} strains at 20 °C whereas at 28 °C, the plants were infected systemically [36]. Similarly, wheat cultivar resistant to wheat streak mosaic virus at 20 °C and 70% RH developed typical mosaic symptoms at 32 °C because its resistance broke down at that temperature [14]. In contrast, in many other virus–host combinations, symptoms were more severe at low temperatures and became less severe with rising temperatures [7, 8, 37]. A defensive response mediated by RNA silencing, which is a temperature-dependent mechanism, was shown to be responsible for symptom production in cassava plants infected with geminiviruses [7]. It is demonstrated that the temperature-dependent expression of the host micro RNAs and target mRNAs were responsible for symptom production in potato plants inoculated with PVY [37]. It is therefore likely that the high DI of PYMoV in black pepper at high temperatures and high RH is either due to low siRNA production under those conditions or due to the temperature-dependent expression of host microRNAs and target mRNAs upon infection with PYMoV or perhaps due to both these mechanisms. It is also possible that the interaction between the viral suppressor and its host counterparts changes with changes in temperature, resulting in much lower gene-silencing activity at high temperatures.

Regular monitoring of black pepper plantations shows that temperature is the primary factor involved in the severity of symptoms. Even under conditions that were

conducive to the expression of symptoms, some cultivars remained symptomless but tested positive for the virus when tested through RT-PCR. The cultivars used in the present experiment represented genotypes of diverse genetic backgrounds and also differed in their response to temperature and RH. Based on the DI, those genotypes in the low-DI category may be considered field tolerant to PYMoV although they need to be evaluated further to ascertain their true response to PYMoV under controlled conditions. Black pepper being a perennial crop, it is also important to ensure that the plants continue to yield well throughout their productive life. The observation that the same nineteen genotypes differed little in their DI in the absence of high temperature and high RH implies that such abiotic factors as temperature and RH are factors that trigger the expression of symptoms. Further, despite the variation in the DI, the concentration of the virus (as determined through real-time RT-PCR) showed no significant difference between plants of a given variety that were exposed to high temperature and high RH and those that were not so exposed, which seems to rule out any correlation between virus titre and the expression of symptoms. Previous researchers [29, 39] also reported that the severity of symptoms does not correlate with the virus titre, suggesting that the disease can be an outcome of specific interactions between the virus and its host.

The incidence of PYMoV in different cultivars of black pepper at a given temperature and RH can be predicted using the formula developed in the present study. Such predictions would help to mitigate the disease by managing the temperature and RH in pepper plantations. In general, the incidence and the severity of PYMoV disease are high in plantations in which black pepper is the sole crop grown in the open—conditions that expose the vines to high temperatures and rain during summer. On the contrary, the incidence and severity are lower in black pepper grown by trailing the vines onto shade trees, a feature of mixed cropping systems in which black pepper is grown along with coffee, tea, arecanut, coconut, or forest trees. Therefore, growing black pepper under shade and irrigating the plants in summer will reduce the incidence and severity of PYMoV. Besides, other abiotic factors such as nutrients and pH of the soil may also contribute to the expression of symptoms in black pepper infected with PYMoV [34]. Accordingly, the role of nutrients, light intensity, vectors, alternative hosts, and mixed infections with viruses such as cucumber mosaic virus in the expression of symptoms also needs to be investigated.

The significant differences between plants grown under natural conditions and under controlled conditions in terms of total carbohydrates and phenols and in lipid peroxidase activity observed in the present experiment indicate the role of these components in symptom development, as was reported in wheat [45], chilli [27], and sugarcane [26] as well. Reduction in the expression of genes involved in pathogenesis due to higher temperatures may be one of the reasons that make the plants susceptible to viruses at high temperatures, as reported in potato [25]. The higher activity of superoxide dismutase observed in all the three cultivars that showed the lowest DI despite being grown under natural conditions (in the polyhouse) may point to the plant's strategy to restrict colonization by the virus.

Although the present study found that DI was higher at higher temperatures and higher RH, the study did not take into account the duration of exposure to a particular temperature and RH, which is an important factor in the expression of symptoms; a future study should fill this gap and should also assess the impact of these factors on yield. A better understanding of the effect of such abiotic factors as temperature and RH on the differences in host–virus interactions both in the field and at the molecular level would help in devising appropriate strategies to manage the disease caused by PYMoV.

Acknowledgements The authors are thankful to the Department of Biotechnology, Government of India, for funding (BT/PR15202/BPA/118/156/2015) and to the Head (Division of Crop Protection) and the Director, ICAR-Indian Institute of Spices Research, Kozhikode, Kerala, India, for the facilities. We also thank K Jayarajan for assistance in statistical analysis and K Anees for assistance in biochemical analysis.

Declarations

Conflict of interest All authors declare that they have no conflict of interest.

Involvement of human participants and/or animals This research did not involve any experimentation on humans or animals.

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