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Aetiology and Control of Black Pepper (*Piper nigrum* L.) Yellow Mottle Virus Disease

D.P.P. De Silva, P. Jones[1] and M.W. Shaw[2]

Plant Pathology Division
Export Agriculture Research Station
Matale

ABSTRACT. *A virus disease of black pepper (Piper nigrum L.) in Sri Lanka was found to be caused by Piper yellow mottle badnavirus (PYMV), measuring 30 × 130 nm. Symptoms of the disease included poor plant growth, mosaic and mottling of leaves, reduction of leaf size and yield. PYMV was transmitted by vectors: the mealy bug, Planococcus citri (Risso) and the lace bug, Diconocoris distanti (Drakes), and through rooted cuttings. PYMV was successfully detected by the polymerase chain reaction (PCR). Nursery surveys showed 0.3%-2.0% infection in black pepper. Plots without weed cover had twice the disease increase (4 ×) in one year period than in weedy plots (2 ×). Frequent observation and roguing of infected vines from 1991-1992 and 1994-1995 gave a reduction in the spread of the disease.*

INTRODUCTION

Black pepper is a long lived perennial vine (usually 50-60 years), belonging to the genus *Piper* and family *Piperaceae*. The main pepper growing countries are India, Malaysia, Indonesia, Brazil and Sri Lanka. Virus diseases of black pepper have been reported since the early 1950's, the first report of this disease was by Bharat (1952) from Vietnam and then in 1959 by Holliday from Sarawak, Malaysia. Subsequently it was reported from all pepper growing countries. During last two decades a virus disease infecting black pepper has become more common in Sri Lankan pepper gardens. Prominent small leaves found on infected vines, initially led it to be called little leaf disease of black

1 Crop and Disease Management Department, Rotamsted Experimental Station, Harpenden, Herts AL5 2JQ, United Kingdom.

2 School of Plant Sciences, Department of Agricultural Botany, University of Reading, Building 2, Earley Gate, Whiteknights Reading, RG6 2AU, United Kingdom.

pepper. Later Lockhart (1993) working in Thailand and Philippines named this disease as *Piper* yellow mottle virus (PYMV) disease.

Since 1979 *Piper* yellow mottle virus (PYMV) disease has become more common in the Sri Lankan pepper gardens and become a threat to pepper production. This report describes the aetiology and possible methods to manage or eradicate the disease.

MATERIALS AND METHODS

Transmission of PYMV by mealy bugs

Non-viruliferous mealy bugs (*Planococcus citri* Risso) were obtained from mealy bug cultures maintained in petri dishes at room temperature. Mealy bugs at the first nymphal stage were used in transmission studies as they are better in transmitting virus than the adults (Thresh, 1978). The first nymphal stage of the mealy bugs were carefully removed with a fine pointed paint brush and transferred on to detached, infected, tender black pepper leaves and allowed acquisition feeding for 24 hrs. Nymphs were then transferred to test plants (black pepper 4 leaf stage seedlings) and allowed inoculation feeding for 2 days; insects were then killed by spraying an insecticide (Dimethoate). The controls, mealy bug nymphs were allowed to feed on healthy detached black pepper leaves for the acquisition feed.

Transmission of PYMV by lace bugs

Investigations were carried out to find the disease transmission with *Diconocoris distanti* (Drakes), *Tingidae* (black pepper lace bug), one of the most common insects found on Sri Lankan black pepper vines. Transmissions were done by following the same method as in mealy bug transmissions, using adult lace bugs. For control plants healthy leaves were used for acquisition feeding. As the 4 leaf stage pepper plants cannot tolerate high numbers of lace bugs, 2-3 lace bugs were transferred to each plant.

PYMV disease transmission by vegetative propagation

In this study the planting material from 4 nurseries (Matale, Walpita, Horombawa and Hollongolla) belonging to the Department of Export Agriculture and two registered private nurseries (Mahawela and Ukuwela) were

surveyed. Rooted cuttings which were ready for release in the *Yala* season (1995) were examined. Two thousand plants were examined for visual symptoms in each of the departmental nurseries except for the Walpita nursery.

PYMV detection in black pepper by electron microscopy

Quick dip method was used in studies of the morphology of virus particles in different preparations. Collodion-carbon coated electron microscope grids were floated on 20 µl drops of PYMV purified extracts in 50 mM Tris - HCl buffer, pH 7.4 for 1 h. Each grid was washed with 1 drop of doubled distilled water and then negatively stained with 1% (w/v) phosphotungstic acid (PTA) before observation in the electron microscope (Jeol, 1200 EX).

PYMV detection in black pepper by polymerase chain reaction (PCR)

Plant material

PYMV disease infected black pepper leaves, *cv.* Panniyur 1, taken from PYMV spread trial, Matale and leaves from mealy bug transmitted black pepper plants were used for the extractions of DNA. Total DNA was prepared by using a modification of the method described by Doyle and Doyle (1990). Sugarcane leaf samples *cv.* Iscambine infected with sugarcane bacilliform virus (ScBV) were kindly provided by S. Samtally, MSIRI, Mauritius for DNA extraction.

Oligo-primers

Specific oligo - primers are not available for PYMV. A number of sequences for badnavirus primers have been published (Braithwaite *et al.*, 1995; R. Briddon, personal communication, 1995) and these were synthesised (Cruachem Ltd.) and tested for their ability to amplify PYMV DNA. Oligonucleotide primer combinations used in PCR were: (i) Badna - T and ScBV R_1 (ii) Badna - 2 and ScBV R_1 (iii) Badna - 3 and ScBV R_1 and (iv) ScBV F_1 and ScBV R_1.

PCR conditions

Different PCR conditions were used to optimise the annealing temperature. Optimal conditions were found to be 5 cycles of: 94°C for 30 sec, 37°C for 30 sec, 72°C for 2 min followed by 25 cycles of: 94°C for 30 sec, 58°C for 30 sec and 72°C for 2 min.

Agarose gel electrophoresis

PCR products were separated in 1% Agarose gel in 40 mM Tris acetate buffer, pH 8.0 containing ethidium bromide and running the gel at 100 V for 80 min.

Monitoring disease incidence

Field plots were established with the *Maha* rains in December 1992 at Matale near to Export Agriculture Research Station, altitude 1,800 ft above sea level. Each plot consisted of 25 plants arranged in a lattice of 5 rows and 5 columns. Plants were 2.4 m apart from both directions. Treatment plots were separated by 3 guard rows of black pepper, *cv.* Panniyur 1, so plots were 9.6 m apart. Each treatment consisted of 24 healthy plants (*cv.* Panniyur 1) with one infected plant at the centre, as the source of inoculum at the initial stage. Each treatment was replicated 3 times and the treatments were,

1. Clean weeded and without insecticide
2. Clean weeded and with insecticide
3. With weed cover and without insecticide
4. With weed cover and with insecticide

All the pepper vines in guard rows had weed cover and no insecticide application. One year after planting, test plants were examined for the disease and thereafter the plants were monitored at the end of every month.

Effect of detecting and roguing infected vines

Pepper vines at Delpitiya mixed cropping model were examined regularly for disease symptoms. Number of infected vines and their locations were recorded, infected vines were uprooted and destroyed at the time of detection to avoid further infection.

Plant counts were compared by the Wilcoxon signed-ranked test. which is based on ranks and does not need the assumption of a normal distribution. Data of years (1990/91 and 1994/95) were compared to find any effect on decrease of disease by roguing infected vines.

RESULTS

Insect transmission of PYMV: by mealy bugs

Disease transmission was successful from infected to healthy black pepper, when the first nymphal stage of the mealy bugs were used as a vector. Of 130 black pepper plants used for mealy bug transmission only 14 plants showed symptoms.

Table 1. Mealy bug transmission of PYMV.

Date	Duration to reach maximum transmission (days)	No. of mealy bugs/test plant	No. of test plants/No. of control plants	No. infected/No. inoculated
12.08.94	-	10	15/5	0/15
22.09.94	-	10	15/5	0/15
05.09.94	98	25	15/5	3/15
08.09.94	124	25	15/5	3/15
08.09.94	-	30	15/5	0/15
20.09.94	71	30	15/5	1/15
14.08.94	90	40	10/5	5/15
10.10.94	92	40	30/10	2/30

None of the control plants had symptoms and 45 plants were used as controls. The Chi-square statistic to test the difference between test plants and controls for mealy bug transmission was $5.25_{(1\ d.f.)}$ and is significant at $P<0.05$. Only the polymerase chain reaction (PCR) technique detected the presence of badnavirus in plants to which PYMV had been transmitted by mealy bugs.

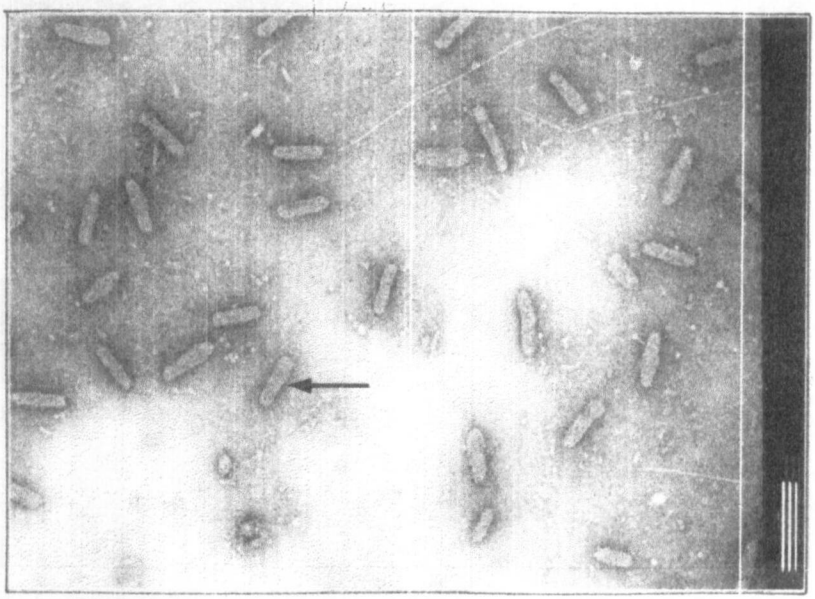


Plate 1. **Electron micrograph showing bacilliform badnavirus particles (bar represents 200 nm).**

Insect transmission of PYMV: by lace bugs

Fleck necrosis, reduction of leaf size and increasing of leaf thickness were observed on the test plants used for lace bug transmission. Virus particles recovered from test plants by purification were confirmed as badnavirus (30 x 130 nm) by electron microscopy.

PYMV disease transmission by vegetative propagation

Most infected plants were observed in Ukuwela private nursery (2%) and fewest in Horombawa nursery (0.3%). Nurseries in the Matale district had higher infection levels than those in Kurunegala district.

PYMV detection in black pepper by Electron microscopy

PYMV particles (30 x 130 nm) were easily seen when 1% neutral PTA was used as a stain (Plate 1).

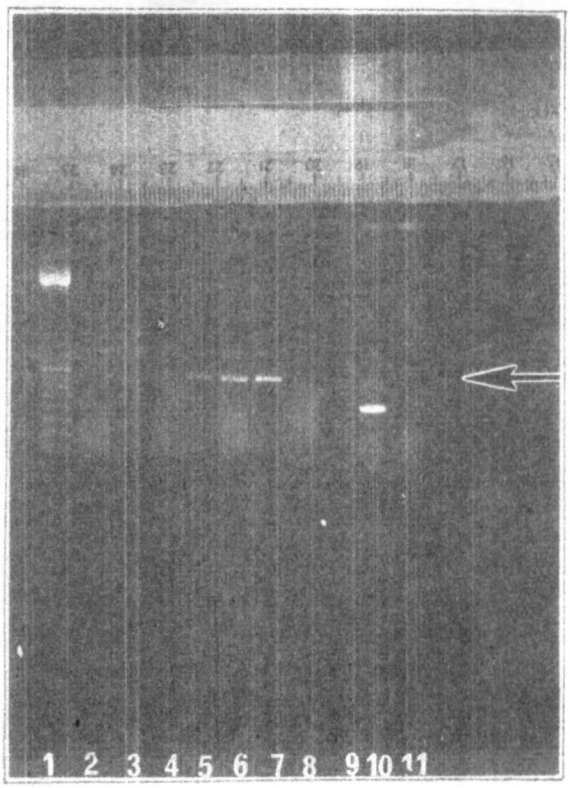
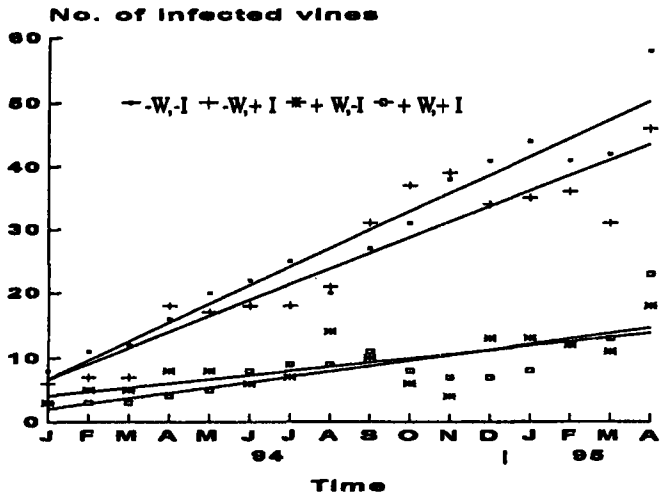


Plate 2. **Detection of PYMV infection in black pepper by polymerase chain reaction. (Lane 4, 5, 6 and 7: Infected pepper extracts with Badna T and ScBV R_1, Lane 2 and 3: Healthy samples, Lane 10: positive control, Lane 11: H_2O control).**

PYMV detection in black pepper by polymerase chain reaction (PCR)

PCR cycles described above, amplified PYMV DNA and ScBV DNA. Plate 2 shows DNA bands of 700 bp size amplified from infected black pepper extracts, no products of similar size were observed in the healthy control. PYMV DNA was only amplified by the primer pair combination Badna-T and



-W, -I : No weeds, no insecticide application
-W, +I : No weeds, with insecticide application
+W, -I : with weeds, no insecticide application
+W, +I : with weeds, with insecticide application

Figure 1. PYMV disease increase in different treatments over time (fitted and observed).

ScBV-R_1. Other primer pairs tested gave no amplification. A few faint bands of 700 bp size were observed with the healthy black pepper extract and similar bands appeared in infected extracts on some occasions. These spurious bands were removed by increasing the annealing temperature to 58°C in the second set of cycles.

The following PCR conditions for PYMV DNA amplification reduced random priming and removed spurious bands. The successful PCR conditions are, 5 cycles of 94°C for 30 sec, 37°C for 30 sec and 72°C for 2 min followed by 25 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 2 min. 700 bp size PCR products were also obtained with the DNA extracts taken from receptor plants used in mealy bug vector studies confirming that PYMV is transmitted by the mealy bug (*Planococcus citri* Risso) and the causal agent as PYMV.

Monitoring disease incidence

The number of infected pepper vines increased with time in all four treatments (Figure 1). A four fold increase of infected vines in one year period was observed with clean weeded and without insecticide application. The treatment with weed cover and without insecticide application had a two fold increase of infected vines per year.

Effect of detecting and roguing infected vines

The difference of removed plant counts at two different periods were analysed by Wilcoxon signed test.

Table 2. Number of rouged pepper vines in two similar periods.

Month	1990/91	1994/95	Difference	Rank	Rank with less frequent sign
September	12	3	9	4	0
November	7	0	7	3	
January	4	3	1	1	
March	6	1	5	2	
May	0	0	0		

In this test zero values have to be excluded. All differences of plant counts were positive and no negative differences. Therefore, total of the ranks with less frequent sign or the test statistic = 0. Although the sample size was 4, this is significant at P=0.062. This indicates that the number of plants need to be rogued has decreased over time.

DISCUSSION

Previously, there had been much debate about the cause of the PYMV disease. Lack of information and its cause hampered the adoption of control

measures against its spread. Our results confirmed PYMV badnavirus (30x130 nm) as the causal agent and this finding supports with the results of the experiments conducted in Thailand (Lockhart, personal communication, 1993). Results of the disease transmission experiments demonstrated that PYMV is transmitted by mealy bugs, lace bugs and through rooted cuttings. Transmission experiments conducted in 1994-1995 confirmed mealy bug, *Planococcus citri* (Risso) and pepper lace bug, *Diconocoris distanti* (Drakes) as vectors of PYMV. This is the first report of *D. distanti* (Drakes) as a virus vector and a vector of PYMV.

Bharat (1952) and Holliday (1959) transmitted a possible virus disease from infected black pepper using the mealy bug *P.citri*. PYMV transmission has been demonstrated in previous studies (Lockhart, personal communication, 1993) but there are no previous reports of recovering PYMV from infected plants by PCR. In this present study PYMV have been recovered from test plants by PCR for the first time. This result confirms PYMV as the cause and *P. citri* as a vector for PYMV.

PYMV badnavirus DNA was detected in nucleic acid extracts of diseased black pepper by PCR amplification of a 700 bp region from the viral genome. Amplification of similar size PCR products from several different DNA extracts of infected black pepper confirm the usefulness of this technique for detecting low concentrations of virus in pepper plants.

The results of experiment on disease spread showed increase of infected plants with time but the results of roguing experiment showed that infection can be reduced by regular observation and removal of infected vines from the field. In weedy treatments without insecticide application a two fold increase of infected vines was observed in one year period while non-weedy plots had a four fold disease increase. Results of nursery surveys conducted in 1995 show 0.3-2% infection in nurseries. In a worst case "scenario" there would be the possibility of including 34 infected plants in every 1,700 plants needed to establish 1 ha of black pepper. If there is a two fold increase of infected plants per year then it is possible to have a 16 fold increase over 5 years which represent 32% infection in a 1 ha block.

CONCLUSIONS

There is evidence that infected planting material is disseminated through nurseries and this should be prevented by using a certification procedure to ensure healthy plants. A scheme may involve indexing of mother

vines by PCR and using the healthy plants as sources for cuttings. It is important to raise rooted cuttings away from infected plants and to screen these against PYMV by PCR before releasing to farmers. After establishing new plantings, frequent inspection and roguing of infected plants are essential to keep the disease under control.

ACKNOWLEDGEMENTS

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REFERENCES

Bharat, H. (1952). Etude Sur le deperissement des poivrieries en Indochine. Archives des recherches agronomiques au Cambodge, Laos, Vietnem. 13: 75-92.

Braithwaite, K.S., Egeskov, N.M. and Smith, G.R. (1995). Detection of sugarcane bacilliform virus using polymerase chain reaction. Plant Disease. 79: 792-796.

Briddon, R. (1995). John Innes Institute, U.K. personal communication.

De Silva, D.P.P. (1996). Studies of black pepper (*Piper nigrum* L.) virus disease in Sri Lanka. Ph.D thesis, University of Reading, U.K.

Doyle, J.J. and Doyle, J.L. (1990). Isolation of plant DNA from plant tissue. Focus 12(1) life technical Inc. pp. 13-15.

Holliday, P. (1959). Suspected virus in black pepper. Commonwealth Phytopathological News. 5: 49-52.

Lockhart, B.E. (1993). University of Minnesota, USA. personal communication.

Thresh, J.M. (1978). Epidemiology of plant virus diseases. pp. 329. *In*: Scott, P.R. and Bainbridge, A. (Eds). Plant disease Epidemiology, Blackwell Scientific Ppublications, United Kingdom.