Evaluation of *Piriformospora indica* against *Piper yellow mottle virus* in Black pepper

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Evaluation of *Piriformospora indica* against *Piper yellow mottle virus* in Black pepper

by

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(2019-11-107)

THESIS

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2021

DECLARATION

I, hereby declare that the thesis entitled "**Evaluation of *Piriformospora indica* against *Piper yellow mottle virus* in Black pepper**" is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Certified that this thesis entitled "**Evaluation of *Piriformospora indica* against *Piper yellow mottle virus* in Black pepper**" is a record of research work done independently by Ms. Lekshmi R. Krishnan under my guidance and supervision and that it has not previously formed the basis for any degree, diploma, fellowship or associateship to her.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Per cent
µg	Microgram
µl	Microlitre
^{0}C	Degree Celsius
Bp	Base pair
CAT	Catalase
Cfu	Colony forming units
Cm	Centimeter
CMV	*Cucumber mosaic virus*
CTAB	Cetyl Trimethyl Ammonium Bromide
DAB	Diaminobenzidine
DAC	Days after colonization
DAT	Days after treatments
DI	Disease incidence
DNA	Deoxyribo nucleic acid
dNTP	Deoxyribo nucleotide triphosphate
EDTA	Ethylenediamine tetraacetic acid
G	Gram
L	Litre
LAMP	Loop mediated isothermal amplification
M	Molar
Mg	Milligram
Ml	Millilitre
Mm	Millimetre
mM	Millimolar
N	Normal

NADPH	Nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium
Ng	Nanogram
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
POX	Peroxidase
PVP	Polyvinyl pyrrolidone
PYMoD	Piper yellow mottle disease
PYMoV	*Piper yellow mottle virus*
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SOD	Superoxide dismutase
TAE	Tris acetate EDTA
VI	Vulnerability index
w/v	Weight/Volume
w/w	Weight/Weight

Introduction

1. INTRODUCTION

Black pepper (*Piper nigrum* L.), popularly known as "black gold" and the "king of spices", belongs to the family Piperaceae and is native to the Western Ghats of India. The dried berries from the vines are of high economic value, making it the world's most traded spice. Black pepper plays a vital role in India's international trade and it is said that the Europeans invaded India primarily for this very spice. The global production of black pepper was 1,103,024 Tons in 2019 (FAOSTAT, 2021). Ethiopia is the world's largest producer and exporter of black peppercorns, producing 34 per cent of the world's total. Other major producers are Vietnam, Brazil, Indonesia, India, China, and Malaysia. India had a production of 48,000 Tons, with Karnataka being the leading producer (21,000 Tons), followed by Kerala (17,000 Tons) and Tamil Nadu (3,000 Tons) in 2018-19 (Spices Board of India, 2021).

Black pepper has one of the longest histories as a sought-after spice, due to its ability to flavour foods, act as a preservative, and add hot to a dish. In addition to its flavour-enhancing properties, black pepper offers many health aids. The pungency and flavour of black pepper are due to the presence of the alkaloid piperine, volatile oil and oleoresins (Zachariah *et al*., 2010). It is mostly used in Indian folk medicine to boost immunity and cure diarrhoea, asthma, prolonged indigestion, gastric illnesses, heartburn, sleeplessness and epilepsy (Fu *et al*., 2010). Peppercorn, a source of vitamin K and the B-complex vitamin group, is high in antioxidants, which protect the human body from cancer and other ailments by removing harmful free-radicals (Dudhatra *et al*., 2012). It is also rich in minerals like manganese, magnesium, potassium, iron, copper, zinc, calcium, chromium, selenium and phosphorus (Meghwal and Goswami, 2012).

However, black pepper is a very challenging crop to cultivate, owing to its high nutrient requirement, sensitivity to soil pH and moisture, susceptibility to pests and diseases and intensive labour requirement. Currently, black pepper production is steadily declining in most of the producing countries due to a number of constraints faced by the farmers. One of

the major constraints is damage caused by pests and diseases. Yield loss due to diseases is more severe compared to pests. There are 17 diseases known to affect black pepper and among them, based on the severity, *Phytophthora* foot rot, slow decline, stunted disease and anthracnose are considered as the most devastating diseases. In Kerala, black pepper has so far been reported to be infected by two major viruses *viz*., *Cucumber mosaic virus* (CMV) and *Piper yellow mottle virus* (PYMoV). These two viruses, often seen together, are the cause of "stunted disease" of black pepper (Lockhart *et al*., 1997). This disease has become a matter of concern to the scientists recently, due to the resultant yield loss. Moreover, farmers are not much aware of the symptoms and the extent of loss that can be caused by these viral diseases, which further complicates their management.

PYMoV (genus: *Badnavirus*; family: *Caulimoviridae*) is a non-enveloped bacilliform virus with double stranded DNA genome of about 7.5 kilo basepair (kbp). The disease has been recorded from different black pepper growing countries and was known in different names, as "little leaf disease" in Sri Lanka (Randombage and Bandara, 1984), "mosaic disease" in India (Prakasam *et al*., 1990), "wrinkled leaf disease" in Malaysia (Kueh and Sim, 1992) and as "stunted disease" in Indonesia (Firdausil *et al*., 1992). At the International workshop on black pepper diseases, 1991 held at Lampung, Indonesia, it was decided to use "stunted disease" as a uniform terminology to include all the viral diseases of black pepper. Though the viral nature of the disease was reported from many of the black pepper-growing countries as early as in 1952 (Barat, 1952; Holliday, 1959), its causal virus remained uncertain for many years. It was only in 1997 that Lockhart *et al*. reported the etiology of the disease. Besides black pepper, PYMoV is also known to infect betel pepper (*Piper betle*), Indian long pepper (*Piper longum*) and many other related species (Siju *et al*., 2008; Bhat *et al*., 2014).

PYMoV-infected black pepper plants are characterized by mosaic, chlorotic mottling, vein clearing, leaf distortion, reduction in leaf size, stunted growth and poor fruit set. As black pepper is vegetatively propagated, the primary spread of the disease occurs through the use of infected cuttings as planting materials. Secondary spread in field

occurs mainly through various species of mealy bugs (*Ferrisia virgata*, *Planococcus citri*, *P. elsiae*) and the black pepper lace bug (*Diconocoris distanti*), which transmit the disease in a semi-persistent fashion (Lockhart *et al*., 1997; de Silva *et al*., 2002; Bhat *et al*., 2003). But vector- mediated transmission is not so efficient under in field level, due to slow movement of mealy bugs. Seed transmission of the virus, though not much important, has also been reported in black pepper (Hareesh and Bhat, 2010).

Black pepper is propagated mainly by vegetative means and hence the use of virus-free plants is necessary to reduce the spread of the disease. So most of the current research on PYMoV focuses on developing rapid and reliable detection techniques that can facilitate the selection of disease-free mother plants for propagation. Though PCR (Polymerase Chain Reaction) and real-time PCR techniques are available for the detection of PYMoV, these methods need sophisticated equipment and are time consuming. Isothermal techniques like Loop-mediated isothermal amplification (LAMP) assay has also been reported for the detection of PYMoV (Bhat *et al*., 2013), but it requires about six primers for amplification, high temperature and longer incubation time. Deeshma and Bhat (2017) reported the occurrence of endogenous-PYMoV, which gets integrated into the genome of black pepper. Atheena *et al*. (2019) devised a PCR and reverse transcription PCR (RT-PCR) based approach to differentiate plants with endogenous and episomal forms of the virus. Mohandas and Bhat (2020) developed a simple, quick and sensitive Recombinase Polymerase Amplification (RPA) assay for the detection of the PYMoV, that can be performed in a resource poor set up, within less than 40 minutes, without using a thermal cycler and is ten times more sensitive than PCR.

PYMoV has now become widely prevalent in black pepper plantations, especially in Wayanad and Idukki districts. The diversity and extent of losses in crop production make it an emerging problem under Indian conditions. The current management strategies of the disease include removal of the severely infected vines and replanting them with healthy vines; but the farmers usually do not prefer this method, especially if the vine has attained certain height and age, and started to yield. Use of resistant varieties is the best way to

manage the disease, but so far no black pepper variety resistant to the virus is available in any of the pepper growing countries. Here comes the possibility of using endophytes for the management of the disease. Endophytes are microorganisms that reside inside plant tissues for a part of their life cycle or their entire lifespan, without any visible symptoms in the plants. They offer a great unexploited potential, which can be used to sustain healthy crops. There are several reports of endophytes being used as potential antagonists against various plant pathogens (Johnson *et al*., 2014; Gill and Tuteja, 2016).

Piriformospora indica (Syn. *Serendipita indica*) (*Sebacinaceae*, *Basidiomycota*) is one such root-colonizing, cultivable endophytic fungus, which was isolated from the rhizosphere of xerophytic shrubs in the Thar Desert of Rajasthan (Varma *et al*., 1999). Since the fungus lacks host specificity, it has a broad host range that includes bryophytes, pteridophytes, gymnosperms, and a large number of angiosperms, and it improves various aspects of plant function (Oelmuller *et al*., 2009; Varma *et al*., 2012). The beneficial interaction of *P. indica* with a variety of agriculturally and horticulturally important crops has resulted in increased growth and resistance/tolerance to biotic and abiotic stresses. (Oelmuller *et al*., 2009; Kumar *et al*., 2012; Johnson *et al*., 2014; Gill and Tuteja, 2016).

P. indica has been reported to be successful in the management of many fungal and bacterial diseases in crop plants. Recently, it has been reported that *P. indica*-primed plants could exhibit improved resistance against even viral diseases like *Tomato yellow leaf curl virus* (Wang *et al*., 2015), *Cowpea aphid borne mosaic virus* (Alex, 2017), *Blackeye cowpea mosaic virus* (Chandran, 2019) and *Bhendi yellow vein mosaic virus* (Chippy, 2020).

So far, the effect of *P. indica* has been exploited for the management of RNA viruses and single stranded DNA viruses only. Its ability to manage double stranded DNA viruses is yet to be unraveled. So, the current study was undertaken with the following objectives:

1. To evaluate the beneficial fungal root endophyte *Piriformospora indica* for the management of PYMoV, which is a double- stranded DNA virus infecting black pepper.

2. To elucidate the role of antioxidants in the tripartite interaction.

Review of literature

2. REVIEW OF LITERATURE

Black pepper (*Piper nigrum* L.) belonging to the family Piperaceae, is an important foreign exchange earner for India. It is used for a variety of purposes, including culinary seasoning, food ingredient and in medication. Although quick wilt / foot rot caused by *Oomycetes* and slow wilt caused by nematodes are the major diseases infecting black pepper, the diseases caused by viruses are also becoming important in recent years due to their systemic nature and the resultant yield loss. Vines once infected become difficult to manage, and in severe stages of the disease, farmers are left with no choice but to remove the infected vines. Lack of virus-resistant black pepper varieties further complicates the problem. Use of beneficial endophytes is a promising alternative for the management of viral diseases in plants.

2.1. INCIDENCE OF PYMoV IN BLACK PEPPER

In India, the virus-like symptoms in black pepper was first recorded in a black pepper nursery at Neriamangalam in Idukki District during 1975 (Pailey *et al*., 1981) and then in Pulpally of Wayanad District during 1978 (Sarma *et al*., 1992). PYMoV was reported for the first time in India by Bhat *et al.* (2003). The disease was sporadic initially, but now it is found in almost all black pepper growing countries in the world.

In Wayanad and Idukki districts of Kerala, 100 percent incidence of PYMoD (Piper yellow mottle disease) was reported from black pepper plantations (Bhat *et al*., 2003). A survey conducted by Bhat *et al*. (2005) in 276 black pepper plantations in 96 major black pepper growing locations in Karnataka and Kerala revealed that the disease incidence was the highest in Wayanad (45.4 per cent), followed by Idukki (29.4 per cent) in Kerala; and in Karnataka the highest incidence was in Kodagu district (14.9 per cent), followed by Hassan (5.2 per cent). Ayisha (2010) surveyed Kollam and Thiruvananthapuram districts and found out that viral diseases are prevalent in both the districts, with a disease incidence ranging from 0-57 per cent. Survey conducted by Arya (2020) in different panchayats of Idukki and

Wayanad districts revealed that the highest disease incidence was in Poothadi (45.5 per cent) of Wayanad and the highest vulnerability index was in Panamaram district (61 per cent) of Wayanad.

Characteristic symptoms such as mild to severe chlorotic mottling, vein-clearing, interveinal chlorosis, reduction in leaf size, leaf puckering and deformation in PYMoV-infected black pepper vines were observed by Lockhart *et al*. (1997) in Sarawak (Malaysia), Sri Lanka, Philippines and Thailand. PYMoV was reported initially in black pepper, but later it was also reported in other *Piper* species like betel pepper (*P. betle*) and Indian long pepper (*P. longum*) (Lockhart *et al*., 1997; Bhat *et al*., 2003; Siju *et al*., 2008). de Silva *et al.* (2002) observed symptoms like leaf distortion, reduction in leaf size, mottling and mosaic, along with stunting of the whole plant, short spike length and poor filling of spikes, leading to yield loss in PYMoV-infected black pepper vines. Early works from India have reported mosaic-like diseases in many black pepper plantations, which were characterized by chlorotic flecking and vein clearing, followed by mottling of leaves leading to inter-veinal chlorosis and curling of leaves, vein banding, vein thickening and green island-like symptoms (Bhat *et al*., 2003). PYMoV-infected betel pepper plants showed mosaic, mottling and reduction in leaf size; whereas Indian long pepper showed mosaic, blisters, dark green patches and leaf distortion (Siju *et al*., 2008). Although the three species (*P. nigrum*, *P. longum* and *P. betle*) showed typical chlorotic mottling symptoms, the severity of the symptoms were maximum in *P. longum*, whereas *P. betle* exhibited only mild symptoms like chlorotic mottles without any leaf deformation.

Though external symptoms are good criteria for the detection of PYMoV, sometimes depending on the season, growth stage and certain other factors, the disease could be difficult to detect visually. Masking of symptoms during monsoon and winter months was also observed in many of the affected black pepper vines (de Silva *et al*., 2002). Under Indian conditions, symptoms are best exhibited during March to May. Furthermore, symptoms are more evident in certain cultivars such as Karimunda, but symptoms are rarely found in varieties such as Panniyur 1, Panniyur 5 and Panchami; and some plants in these

varieties may even remain symptomless for years (Bhat *et al*., 2012). Surprisingly, plants constantly exposed to high temperatures of around 35°C displayed greater symptom expression, however when these plants were continually cultivated at a lower temperature of roughly 22-28°C, the symptoms were barely noticeable. Furthermore, when asymptomatic plants cultivated at 22-28°C were transferred to 35°C, both disease severity and virus titer increased significantly within 15-30 days (Umadevi *et al*., 2016). It is unknown whether this type of symptom manifestation is caused by enhanced viral replication or by the activation of endogenous PYMoV into infective PYMoV.

2.2. MOLECULAR DETECTION OF PYMoV

PCR Amplification

PCR is the most widely used technique for the detection of plant viruses, as it can amplify target DNA or RNA present even in extremely low levels (5-10 ng). It is an *in-vitro* technique based on DNA polymerization reaction and consists of repeated cycles of heating and cooling for melting, annealing and enzymatic replication of DNA using thermo-stable DNA polymerase (Taq polymerase), specific primer sequence (complementary to the sequence flanking the target region) and dNTPs. Using PCR, a specific sequence of DNA can be amplified by as many as one billion times. PCR-based detection method of PYMoV infecting black pepper was reported from Sri Lanka (de Silva *et al*., 2002) as well as from India (Bhat *et al*., 2009). The PCR technique reported by Bhat *et al*. (2009) using PYMoV specific primers designed based on the ORF III sequence of PYMoV reported from India, could efficiently detect PYMoV infection in black pepper plants of all the varieties tested.

2.3. EVALUATION OF *P. indica* -COLONIZED BLACK PEPPER CUTTINGS AGAINST PYMoV

2.3.1. Maintenance of *P. indica*

P. indica can be propagated axenically on various complex and minimal substrates in the absence of host plants (Varma *et al.,* 1999). *P. indica* chlamydospores germinate and

form hyphae which look like a string of beads (monilioid hyphae), which after a few days, develop chlamydospores again. Hill-Kaefer complex medium (Aspergillus medium), containing 25 different macro- and micro-ingredients is the most commonly used medium for culturing *P. indica* (Hill and Kaefer, 2001). Using this complex agar medium, *P. indica* could cover a nine cm Petri-dish with hyphae in three weeks. Kumar *et al*. (2011) could obtain optimum growth and sporulation of *P. indica* when peptone, yeast extract and soybean meal were added to Hill-Kaefer medium at a concentration of 2.0 g L^{-1} each. It was also reported that the fungus grows profusely upon shaking in Hill-Kaefer broth medium. The temperature range for optimum growth was found to be 25-35°C, with the optimum temperature being 30°C, and a pH range of 4.8-6.8, with an optimum of 5.8. The colonies were large and small, showing sea urchin-like radial growth.

Johnson *et al*. (2013) sub-cultured five mm diameter *P. indica* plugs every four weeks to the middle of fresh combined Kaefer medium and incubated in the dark at 22- 24°C, to maintain virulence. They also reported that the fungus loses its root colonization efficiency on repeated sub-culturing, thus necessitating the need for periodical inoculation of the fungus onto the roots of host plants and then re-isolating it from the internally colonized roots. Jisha *et al*. (2019) reported that potato dextrose broth (PDB) and potato dextrose agar (PDA) showed the best and maximum growth of *P. indica*. They could observe mat-like growth of *P. indica*, with several concentric rings in PDA and as globular balls in PDB.

Osman *et al.* (2020) optimized a simple vegetable-juice medium, which is superior to the Kaefer medium, with respect to biomass production in liquid medium and hyphal growth on agar plates. They reported that the agar plates were completely covered with the fungal mycelium on eight days of post inoculation, compared to three weeks in Kaefer medium.

2.3.2 Co-cultivation of Rooted Cuttings of Black pepper with *P. indica*

Pedrotti *et al*. (2013) isolated chlamydospores from 3-6 weeks old *P. indica* grown in Kaefer medium and washed three times by centrifugation at 3000 G for 10 minutes using

30 ml of 10mM $MgCl_2$ solution. 15 ml of a 5×10^5 spore ml^{-1} of *P. indica* spore suspension was then used to inoculate *Arabidopsis thaliana* seeds sown in sterile 0.5 ml tubes containing one per cent phytoagar medium prepared with Murashige and Skoog basal salt mixture.

In-vitro experiments have shown that even very small amounts (2.5 ml L^{-1} of medium) of culture filtrate are sufficient to promote root and shoot growth in crops like *Triticum aestivum, Cicer arietinum, Phaseolus vulgaris, Brassica campestris* and *Broccoli* (Varma *et al*., 2012). Johnson *et al.* (2013) described *in-vitro* co-cultivation of *Arabidopsis* seedlings (nine to twelve weeks old) and *P. indica* plugs (four weeks old) in Petri-dishes containing modified PNM medium.

Das *et al.* (2014) successfully used potting mixture composed of unsterilized sand (pH 7.2 and an electrical conductivity of 0.11 dsm), soil and compost (1:1:0.25 w/w) for the co-cultivation of rooted cuttings of *Coleus forskohlii* with *P. indica.* Polythene bags were filled with a "sandwich layer" of the potting mixture with 2 per cent of fungal biomass (w/w). Anith *et al*. (2018) thoroughly mixed the mycelia of *P. indica* harvested from potato dextrose broth (PDB) with sterile vermiculite to achieve a final concentration of one percent (w/v) of the fungal mycelial mass. They placed 50 g of the fungal inoculum prepared in sterile vermiculite into a small cavity made in the potting medium in the earthen pots and rooted cuttings of black pepper were then planted in the cavity above the inoculum, so that when the roots spread further, they will come in direct contact with the fungal inoculum. *P. indica* colonization could be observed when the roots of three weeks old tomato seedlings raised on perlite were soaked in the *P. indica* inoculum suspension (3×10^5 cfu ml^{-1}) for 24 hours (Hallasgo *et al*., 2020).

Druege *et al.* (2007) reported that inoculation of the rooting substrate with *P. indica* could promote the development of adventitious roots in Poinsettia and Pelargonium. But, Anith *et al*. (2011) could not observe colonization in stem cuttings of black pepper planted in sterile sand amended with *P. indica*, even after one month, which may possibly be due to the low survival ability of the fungus in the sterile sand medium till the roots emerge; which takes 20–25 days. They also reported that when rooted cuttings of black pepper grown on *P.*

indica-amended sterile sand medium, colonization by the fungus could be observed on the seventh day after inoculation.

2.3.3. Root Colonization Efficiency of *P. indica* on Black pepper

Anith *et al.* (2011) could obtain 16.7 per cent root colonization by *P. indica* on rooted cuttings of black pepper on drenching mycelial suspension, 22.4 per cent on incorporation of one per cent inoculum-soil mixture and 94.3 per cent on transplanting into one per cent soil-inoculum mixture.

2.3.4. Evaluation of *P. indica*-Colonized Black pepper Cuttings against PYMoV

The first indications for the biotic stress protection by *P. indica* were obtained in barley, in which the fungus-colonized plants were found to be more resistant to *Blumeria graminis* (causing powdery mildew) infection in shoots and *Fusarium culmorum* (causing crown rot) in roots (Waller *et al*., 2005). Similar findings were obtained in many other plant species and pathogen isolates, for instance, *Pseudocercosporella herpotrichoides* in wheat (*Triticum aestivum)*, *Fusarium verticillioides* in maize (*Zea mays)*, *Verticillium dahliae* in tomato (*Lycopersicum esculantum*) and many more (Serfling *et al*., 2007; Kumar *et al*., 2009; Fakhro *et al*., 2010).

First report of the successful use of *P. indica* against a viral disease was by Fakhro *et al*. (2010), in which they were able to curb the concentration of *Pepino mosaic virus* (PepMV) in tomato plants colonized by *P. indica*. They could observe that the concentration of PepMV decreased over time (14, 41, and 57 days after inoculation) in the upper most leaves, but the decrease was always 10-20 per cent higher in tomato plants colonized by *P. indica*, than in the non-colonized controls. They also found out that light intensity plays a crucial role in *P. indica*-mediated resistance, as the colonized plants kept under high light intensity had a lower concentration of the virus, than those kept under shaded conditions.

Wang *et al.* (2015) inoculated *P. indica* onto the roots of a *Tomato yellow leaf curl virus* (TYLCV)-resistant tomato cultivar T07-4 and a susceptible cultivar T07-1, to study the

effects of *P. indica* inoculation on resistance to TYCLV. The result was that, the resistant cultivar T07-4 inoculated with *P. indica* as well as the non-inoculated plants showed no TYLCV disease symptoms and grew well in the field filled with viruliferous colonies of *Bemisia tabaci*, whereas in the case of susceptible cultivar T07-1, *P. indica* inoculated plants showed TYCLV incidence and disease index of 7 per cent and 0.47 respectively, while for the non-inoculated T07-1 plants it was 33 per cent and 1.72 respectively.

Studies conducted by Alex (2017) revealed that *P. indica*-primed cowpea plants were less vulnerable to *Blackeye cowpea mosaic virus* (BICMV) infection, and inhibited more than 50 per cent of infection by the virus. Inoculation with *P. indica* could reduce BICMV infection not only in cowpea, but also in the local lesion host, *Chenopodium amaranticolor* by 68 per cent, over the un-inoculated control plants (Chandran, 2019).

Pot culture studies conducted to evaluate the effect of *P. indica* priming against the natural incidence of *Bhendi yellow vein mosiac virus* (BYVMV) revealed that *P. indica*-priming significantly reduced vulnerability index (V. I.) by 56 per cent and disease incidence (D. I.) by 57 per cent, over the un-inoculated control. Also, pre- and post-inoculation of bhendi with the fungus, followed by artificial inoculation of BYVMV by grafting, could reduce the V. I. by 56 and 24 per cent respectively (Chippy, 2020).

2.4. ELUCIDATING THE ROLE OF REACTIVE OXYGEN SPECIES (ROS) AND ANTIOXIDANT ENZYMES IN THE TRIPARTITE INTERACTION

Plants produce several types of antioxidant scavengers to deal with ROS produced during disease progression (Alscher *et al*., 1997). ROS can exist in both radical and non-radical forms and are produced during typical metabolic activities, such as electron transport chains in chloroplasts and mitochondria. On the other hand, adverse conditions such as abiotic and biotic stressors can greatly speed up the generation of ROS at the cellular level (Rasool *et al*., 2013). Radical forms of ROS majorly include superoxide radicals, alkoxy radicals and perhydroxy radicals; whereas non-radical forms include hydrogen peroxide and

singlet oxygen. Compared to non-radicals, radical forms of ROS are more toxic due to their highly reactive nature (Sewelam *et al*., 2016).

Plants have evolved enzymatic and non-enzymatic antioxidant systems that work together to detoxify ROS (Foyer and Noctor, 2005). ROS scavenging systems in plants include enzymatic systems such as sodium superoxide dismutases (SODs), glutathione peroxidases (GPXs), ascorbate peroxidases (APXs), catalases (CATs), glutathione-S-transferases (GSTs) and glutathione reductases (GRs), as well as non-enzymatic systems such as glutathione (GSH) (Gill and Tuteja, 2010). There is a steady-state balance between ROS buildup and the antioxidant defense mechanism in plant cells (Hasanuzzaman *et al*., 2012). In order to carry out proper redox biology reactions and to regulate numerous processes essential for plants such as growth and development, maintaining an optimum ROS level in the cell is crucial. This intermediate level is maintained by the balance between ROS production and ROS scavenging (Hasanuzzaman *et al*. 2019). During stressful situations, however excessive ROS generation disrupts the balance and causes cellular damage, resulting in programmed cell death (PCD) and a reduction in plant yield (Raja *et al*., 2107). *P. indica* and other fungal endophytes enable plants to more efficiently scavenge ROS or prevent ROS generation under stress conditions.

P. indica–induced abiotic stress tolerance was shown to be associated with elevated levels of ascorbate and a high ascorbate/DHA ratio, along with increased dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) activities in plant roots (Baltruschat *et al.* 2008; Vadassery *et al.* 2009; Waller *et al.* 2005).

P. indica-mediated systemic resistance against *Blumeria graminis* causing powdery mildew in barley was associated with elevated GR activity, suggesting that higher GR activity maintains an enhanced level of reduced glutathione which is involved in maintaining antioxidant capacity (Waller *et al*., 2005). A study conducted by Kumar and associates (2009) revealed that *P. indica*-colonized maize plants showing resistance to *Fusarium verticillioides* had increased levels of CAT, GR, GSH and SOD activities when compared to control plants.

It has already been reported that CAT attenuate the elicitation of plant defense response by scavenging H_2O_2 (Wu *et al*., 1997), which supports the role of CAT in disease suppression in *P. indica*-colonized plants. Also, the increased SOD activity in the case of *P. indica-c*olonized plants might have resulted in the accumulation of more H_2O_2, due to which less infection of *Fusarium* was observed in maize plants, therefore plants recover their biomass. In contrast to this, Harrach *et al.* (2013) could observe a significant reduction in the activities of antioxidant enzymes SOD, APX, GR, DHAR, and MDHAR in *P. indica*-colonized barley, challenged with *Fusarium culmorum*.

Results similar to Kumar and associates (2009) were obtained by Narayan *et al.* (2017), when they used *P. indica* for the management of *Botrytis cinerea* in bengal gram, in which inoculation of the plants with *P. indica* strongly stimulated the activities of the antioxidative enzymes both in roots and shoots. In case of roots there was a 34 fold increase in GST activity, whereas in shoots the increase was 48 folds, compared to control plants. The role of GST in reducing necrosis by reclaiming lipid hydroperoxides which are produced during the peroxidation of membrane (an indication of early damage by ROS) has already been proved (Gullner and Komives, 2001), which substantiates the role of increased GST levels in *P. indica*-colonized plants as observed by Narayan *et al.* (2017). They could also observe an increase in total SOD activity in the colonized plants as compared to the control plants.

When *P. indica*-colonized Chinese cabbage (*Brassica campestris* L. ssp. *Chinensis*) plants were exposed to polyethylene glycol to simulate drought stress, the activities of POX, CAT, and SOD in the leaves were up-regulated within 24 hours, delaying the drought-induced reduction in photosynthetic efficiency and degradation of chlorophylls and thylakoid proteins (Sun *et al*., 2010). A study conducted by Akum *et al.* (2015) demonstrated that the expression of candidate effector (PIIN_08944) of *P. indica* decreased the ROS burst activated by flg22 (a PAMP (pathogen-associated molecular pattern) derived from bacterial flagellin) and chitin in barley. Nassimi and Taheri (2017) found that colonizing rice roots with *P. indica* resulted in higher growth, a delay in *Rhizoctonia solani* infection, and a

reduction in the severity of sheath blight. They discovered that lower levels of H_2O_2 and greater SOD activity were related with lower disease severity. Matsuo *et al*. (2015) reported that *P. indica* actively blocks ROS accumulation by activating ROS-scavenging genes in the *Arabidopsis* plants with Redox Responsive Transcription Factor 1 (*RRTF1*) knockout lines (rrtf1), wild type (WT) as well as over-expressor (oe) lines, with pronounced effects on the oe lines. They also confirmed that stress-exposed oe lines could survive better in the presence of *P. indica,* and this is associated with a strong reduction of the ROS level.

Alex (2017) and Chandran (2019) confirmed that *P. indica*-primed cowpea plants exhibited reduced incidence of *Blackeye cowpea mosaic virus* (BICMV) due to increased production of CAT, POX and peroxidase enzymes. A study conducted by Chippy (2020) revealed that *P. indica* conferred resistance to *Bhendi yellow vein mosaic virus* (BYVMV), which was attributed to an increase in CAT, POX, polyphenol oxidase and ascorbic acid oxidase.

Materials and Methods

3. MATERIALS AND METHODS

All the laboratory experiments and pot culture studies connected with the research work entitled "Evaluation of *Piriformospora indica* against *Piper yellow mottle virus* in black pepper" were carried out in Department of Plant Pathology, College of Agriculture, Vellayani during 2019-2021.

3.1. MAINTENANCE OF PYMoV-INFECTED PLANTING MATERIALS OF BLACK PEPPER

A survey was conducted in the Instructional Farm of College of Agriculture, Vellayani, for the collection of PYMoV-infected rooted cuttings of black pepper.

Incidence of PYMoD in the surveyed nursery was calculated as;

$$\text{Disease incidence (\%)} = \frac{\text{Number of infected plants}}{\text{Total number of plants observed}} \times 100$$

Disease score chart was prepared for PYMoD in black pepper, as per the 0-5 scale developed by Ayisha (2010) with modifications (Plate 1).

0 - No symptoms
1 - Very light mottling of young leaves
2 - Severe mottling of leaves
3 - Curling and distortion of leaves
4 - Reduction in leaf size
5 - Stunted growth

Disease severity in terms of vulnerability index was calculated separately for the available varieties, as described by Bos (1982). The varieties were then categorized into five grades based on their comparative resistance to PYMoV (Table 1) (Ayisha, 2010).

$$\text{Vulnerability Index} = \frac{0n_0 + 1n_1 + \ldots + 5n_5}{n_t\,(n_c - 1)}$$

where; $n_0, n_1 \ldots\ldots, n_5$ - Number of plants in the categories 0,1..., 5

n_t - Total number of plants

n_c - Total number of categories

The characteristic symptoms produced by PYMoV in black pepper plants were recorded and the collected samples were maintained in insect-proof conditions.

3.2. MOLECULAR DETECTION OF PYMoV

Total genomic DNA was isolated by CTAB (Cetyl trimethyl ammonium bromide) method as per the protocol described by Doyle and Doyle (1990). For this, 200 mg of plant tissue was ground into fine powder using liquid nitrogen. Two ml of CTAB buffer (Appendix I) preheated to 65°C, a pinch of polyvinyl pyrrolidone (PVP) and two per cent β-mercaptoethanol were added to this and homogenized. One ml of the extract was taken in eppendorf tubes and incubated in a water bath at 65°C for 30 minutes. The samples were then centrifuged, the supernatant was transferred to a fresh tube and 0.5 ml of chloroform: isoamyl alcohol (24:1) was added to it and mixed well. The tubes were again centrifuged and the upper aqueous phase which contains the DNA was collected and transferred to a fresh tube. To this, equal volume of ice-cold isopropanol was added, mixed well and incubated overnight at 4°C to precipitate DNA. The tubes were centrifuged at 10,000 rpm for seven minutes. The supernatant was discarded and the DNA pellet was washed using 100 µl of 70 per cent ethanol and centrifuged at 10,000 rpm for five minutes, to remove salt contamination. The ethanol was discarded and the pellet was air dried and dissolved in 50 µl of Tris-EDTA (TE) buffer (Appendix I).

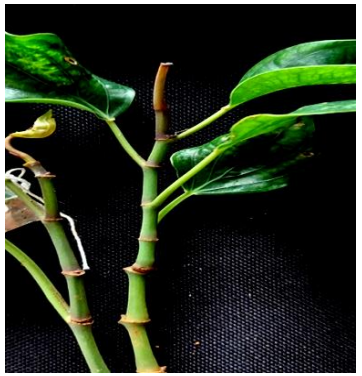
(a)

(b)

(c)

(d)

(e)



(f)

Plate 1: Score chart for PYMoD: (a) No symptom (b) Light mottling of leaves (c) Severe mottling of leaves (d) Crinkling and distortion of leaves (e) Reduction in leaf size (f) Stunted growth

Agarose gel electrophoresis (0.8 per cent) was performed to confirm the presence of genomic DNA. For this, 0.5 g agarose was added to 50 ml of TAE (Tris Acetate EDTA) buffer (1X) (Appendix II) and melted. On cooling, three µl of ethidium bromide was added to it and casted on a horizontal gel electrophoresis unit (Biorad, USA). Once the gel has solidified, the comb was removed and the wells were loaded with sample DNA mixed with 6X gel loading dye, in 6:2 ratio. 1 kb DNA ladder (GeNei, India) was loaded into one of the wells, to serve as molecular marker. The gel was run at 65 Vcm^{-1} in 1X TAE buffer for two hours. The gel was then visualized using a UV trans-illuminator system (Biorad, USA) and documented using a Gel documentation system (Gel Doc TM XR+, Biorad, USA).

Quality of the DNA isolated was checked using bio-spectrophotometer (Eppendorf, Germany). For this, five µl of DNA was dissolved in three ml of 1X Tris-EDTA (TE) buffer in a cuvette and mixed thoroughly, and three ml TE buffer alone was used as blank. The absorbance readings at 260 nm and 280 nm were recorded. The ratio of A_{260} to A_{280} indicates the quality of DNA isolated. Good quality DNA will have an A_{260}/ A_{280} value in the range of 1.8 to 2.

The quantity of DNA isolated was calculated as:

Concentration of DNA ($\mu g\ ml^{-1}$) = A_{260} x 50 ng μl^{-1} x dilution factor

The isolated DNA was subjected to PCR, using primer pair specific to ORF III of PYMoV (Table 2). The PCR reaction mix was prepared as detailed in Table 3, and the mix was run in a thermocycler, under the specified conditions (Table 4).

The amplified products were checked by running in 1.2 per cent agarose gel prepared in 1X TAE buffer (0.6 g agarose in 50 ml TAE buffer), containing 1.8 µl ethidium bromide. Five µl of PCR product was mixed with two µl of 6X loading dye and loaded into the wells. One kb DNA ladder was used as molecular marker, and the gel was run for two hours at 65V. After completion of the run, the gel was visualized and documented.

Table 1: Grading and categorization of black pepper varieties based on vulnerability index to PYMoV

Grade	Vulnerability Index	Category
I	0	Resistant
II	1-25	Moderately resistant
III	26-40	Moderately susceptible
IV	41-50	Susceptible
V	>50	Highly susceptible

Table 2: Oligonucleotide primers specific to ORF III, used for the detection of PYMoV

Primer name	Sequence (5´- 3´)	Amplicon size (bp)	Reference
AIB 104 AIB 105	CTATATGAATGGCTAGTGATG TTCCTAGGTTTGGTATGTATG	400	Bhat *et al*., 2009

Table 3: Reaction mix for PCR (25 µl)

Components	Quantity
10X PCR buffer	2.5 µl
25 mM $MgCl_2$	2.5 µl
10 mM dNTPs	0.5 µl
10 µM Forward primer	0.5 µl
10 µM Reverse primer	0.5 µl
Template DNA	25 ng
Taq polymerase (5 units μl^{-1})	0.3 µl
Sterile distilled water	-

Table 4: PCR conditions for the amplification of ORF III sequence of PYMoV

Function	Temperature (°C)	Time (minutes)	Number of cycles
Initial denaturation	94	3	1
Denaturation	94	0.5	34
Primer annealing	56	1	
Primer extension	72	1	
Final extension	72	10	1

3.3. EVALUATION OF *P. indica*-COLONIZED BLACK PEPPER CUTTINGS AGAINST PYMoV

3.3.1. Maintenance of *P. indica*

The pure culture of *P. indica* was obtained from Department of Plant Pathology, College of Agriculture, Vellayani (from Prof. Dr. Ralf Oelmuller, Institute of General Botany and Plant Physiology, Friedrich-Schiller University, Jena, Germany; and originally from Prof. Dr. Ajit Varma; No. INBA3202001787). Five mm discs with actively growing hyphae of *P. indica* were transferred to Petri-dishes containing Potato Dextrose Agar (PDA) medium (Appendix II) and were incubated in an inverted position at room temperature. The cultures were continuously sub-cultured once in 15 days. Three to four fully grown agar discs each, were inoculated onto 250 ml Erlenmeyer flasks containing 100 ml Potato Dextrose Broth (PDB) and incubated at room temperature for 20 days.

Coirpith-dried farmyard manure (1:1) mixture, amended with two per cent gram flour was used for the mass-multiplication of *P. indica* (Jojy *et al.*, 2020). The medium was moistened and sterilized at 121°C for 20 minutes, consecutively for three days. The mixture was then filled in plastic trays. Mycelium of *P. indica* grown in PDB was harvested by filtering through a clean muslin cloth and washed with two changes of sterile water. It was then thoroughly mixed with the mixture filled in trays @ 10^6 cfu g^{-1} and moistened with sterile water. The trays were then wrapped with cling film and kept undisturbed at room temperature for one week.

3.3.2. Co-cultivation of Rooted Cuttings of Black pepper with *P. indica*

Rooted cuttings of black pepper variety Panniyur 1 were removed from polybags, without disturbing the root system. The roots were thoroughly washed in tap water to remove the adhering soil and other contaminants. They were surface sterilized by dipping in 0.1 per cent mercuric chloride solution for one minute, and washed in tap water. Then they were dipped in 0.05 per cent bactericide (amoxicillin) solution for 30 minutes, followed by

washing with tap water. The rooted cuttings were planted in polybags filled with the *P. indica* mass-multiplied media.

3.3.3. Checking Root Colonization by *P. indica*

Roots of the co-cultivated plants were collected, washed thoroughly in tap water and were cut into one cm long pieces. The root bits were transferred to test tubes containing five ml of freshly prepared KOH solution (10 %) and kept in a water-bath at 65°C for five minutes. They were washed with sterile water, followed by neutralization with one per cent HCl for five minutes and again washed in tap water. The roots were stained with trypan blue in 0.5 per cent lactophenol (Appendix III) for two minutes, and were observed under microscope ((Leica-ICC50 HD, USA) to check the root colonization.

3.3.4. Evaluation of *P. indica*-Colonized Black pepper Cuttings against PYMoV

A pot culture experiment was conducted in Department of Plant Pathology, College of Agriculture, Vellayani, for the evaluation of *P. indica* against PYMoV in black pepper variety Panniyur 1, to find out the effect of *P. indica* in managing PYMoV in black pepper. The experiment was laid in Completely Randomized Design (CRD), with nine treatments and seven replications per treatment (Plate 2).

T1: *P. indica*-colonized cuttings alone
T2: PYMoV infected cuttings alone
T3: PYMoV infected cuttings + *P. indica* colonization
T4: *P. indica* colonization on healthy rooted cuttings, followed by grafting PYMoV- infected scion on the same day
T5: *P. indica* colonization on healthy rooted cuttings, followed by grafting PYMoV- infected scion after two days
T6: *P. indica* colonization on healthy rooted cuttings, followed by grafting PYMoV- infected scion after seven days
T7: *P. indica* colonization on healthy rooted cuttings, followed by grafting of PYMoV-infected scion after 15 days

T8: *P. indica* colonization on healthy rooted cuttings, followed by grafting PYMoV- infected scion after 30 days

T9 : Absolute control

Five months old rooted cuttings of black pepper var. Panniyur 1 were used for the pot culture study. For the pre-inoculation treatment (T3), rooted cuttings of black pepper which were already infected by PYMoV were colonized with *P. indica*. For the post-inoculation treatments (T4, T5, T6, T7 and T8), the roots of healthy cuttings of black pepper were sterilized as mentioned earlier and were planted in nursery bags containing *P. indica* mass-multiplied medium. After three weeks, they were carefully removed from the nursery bags, checked for *P. indica* colonization and were planted in large-sized grow bags containing normal potting mixture. The PYMoV-infected scions showing moderate symptoms were grafted onto the *P. indica*-colonized healthy rooted cuttings, at 0, 2, 7, 15 and 30 days after co-cultivation with *P. indica*. For this, a wedge-shaped cut was made at the base of the PYMoV-infected scion and it was inserted into a cleft made on the *P. indica*-colonized healthy rootstock and the union was tied firmly using a grafting tape.

In order to select the best post-inoculation treatment, total genomic DNA was isolated from the newly emerged leaves of the treatments T4, T5, T6, T7 and T8. The DNA was quantified using spectrophotometer and 1 ng of DNA from each of the treatments was used as template DNA for PCR amplification using primer pair specific to ORF III of PYMoV. The treatment that showed the least amplicon size was selected as the best treatment and was used as the only post-inoculation treatment for the further study. The plants were then observed for symptom development, vulnerability index, virus titer, *P. indica* colonization, number and size of leaves, number of branches and length of shoots at monthly intervals.

(a) **(b)**

Plate 2: (a) General view of pot culture experiment for the evaluation of *P. indica* against PYMoV in Panniyur 1 variety of black pepper (b) *P. indica*-colonized healthy rooted cutting of Panniyur 1 grafted with PYMoV-infected scion

3.4. ELUCIDATING THE ROLE OF ROS AND ANTIOXIDANT ENZYMES IN THE TRIPARTITE INTERACTION

Biochemical changes were analyzed for T1, T2, T3, the best treatment from T4 to T8 and T9 at 15, 45, 60 and 90 days after the treatments. Total chlorophyll, total soluble protein, antioxidant enzymes *viz*. catalase, peroxidase, phosphatase, superoxide dismutase, glutathione reductase and glutamate synthase were estimated. Presence of superoxides was detected using nitroblue tetrazolium chloride (NBT) staining and hydrogen peroxide by diaminobenzidine (DAB) staining.

3.4.1. Estimation of Chlorophyll

Chlorophyll was estimated as per the procedure described by Arnon (1949). One gram of leaf tissue was homogenized using 20 ml of 80 per cent acetone, in a pestle and mortar and centrifuged. The supernatant was collected in a 100 ml volumetric flask and the above steps were repeated till the residue turned colourless. The volume in the flask was made up to 100 ml using 80 per cent acetone. The absorbance of the samples was read at 645 nm and 663 nm using a spectrophotometer, against 80 per cent acetone as blank. Chlorophyll a, b and total chlorophyll were calculated using the following equations and were expressed as mg chlorophyll per g of tissue.

$$\text{Chlorophyll a (mg g}^{-1}) = 12.7\ (A_{663}) - 2.69\ (A_{645}) \times \frac{V}{1000 \times W}$$

$$\text{Chlorophyll b (mg g}^{-1}) = 22.9\ (A_{645}) - 4.68\ (A_{663}) \times \frac{V}{1000 \times W}$$

$$\text{Total chlorophyll (mg g}^{-1}) = 20.2\ (A_{645}) + 8.02\ (A_{663}) \times \frac{V}{1000 \times W}$$

where, V - final volume of chlorophyll extract in ml

W - weight of leaf sample in g

3.4.2. Estimation of Total Soluble Protein

Total soluble protein was estimated as per the method described by Bradford (1976). In brief, one gram of leaf sample was ground with 10 ml of 0.1 M sodium acetate buffer (pH 4.7) (Appendix IV) using a pestle and mortar. The homogenate was centrifuged at 5,000 rpm at 4°C for 15 minutes and the supernatant obtained was used as the crude enzyme extract for analysis. The reaction mixture consisted of 0.5 ml enzyme extract, 5 ml dye solution (1 X) (Appendix IV) and 0.5 ml distilled water. One ml of distilled water in 5 ml of 1 X dye solution served as blank, and the absorbance was read at 595 nm. Standard curve for protein was plotted using bovine serum albumin (BSA) and the protein content in the sample was expressed as mg BSA equivalent of soluble protein per gram of tissue.

3.4.3. Estimation of Catalase

Catalase activity was estimated as per the procedure described by Luck (1974). One gram of leaf tissue was ground in 20 ml of 0.0067 M phosphate buffer pH 7 (assay buffer diluted ten times) (Appendix V) at 4^0C and centrifuged at 5,000 rpm for 15 minutes at 4^0C. The sediment was stirred with cold phosphate buffer and the extraction step was repeated. The supernatants were combined and used as crude enzyme extract for the analysis. The experimental cuvette was filled with 40 µl enzyme extract and three ml H_2O_2-PO_4 buffer (Appendix V) while the control cuvette contained 40 µl enzyme extract and three ml H_2O_2-free PO_4 buffer. The time taken for the change in absorbance by 0.05 at 240 nm was recorded for calculating the enzyme units per ml of the enzyme extract.

3.4.4. Estimation of Peroxidase

Peroxidase activity was estimated as per the procedure described by Srivastava (1987). One gram of leaf tissue was ground in five ml of 0.1 M sodium phosphate buffer (pH 6.5) (Appendix VI) with a pinch of PVP in a chilled pestle and mortar. The homogenate was centrifuged at 6,000 rpm for 15 minutes and the supernatant was used as the enzyme extract. One ml of 0.05 M pyrogallol and one ml of one per cent H_2O_2 were taken in a

cuvette and the absorbance was adjusted to zero at 420 nm. One ml of the enzyme extract was added to the cuvette and the change in absorbance was recorded at 30 seconds intervals upto 180 seconds.

3.4.5. Estimation of Glutathione Reductase

Glutathione reductase activity was estimated as per the procedure described by Smith *et al.* (1988). One gram of leaf sample was homogenized with 10 ml of 0.1 M potassium phosphate buffer (pH 7.5) (Appendix V) containing 0.5 mM ETDA. The homogenate was filtered through cheese cloth and the filtrate was centrifuged at 20,000 rpm for 10 minutes. The supernatant was used for the assay. The reaction mixture (two ml) contained one ml of 0.2 M potassium phosphate buffer (pH 7.5) containing one mM EDTA, 0.75 ml distilled water, 0.1 ml two mM NADPH, 0.05 ml enzyme extract and 0.1 mL 20 mM glutathione (oxidized). The reaction was initiated by the addition of glutathione (oxidized) and the decrease in absorbance at 340 nm was recorded. One unit of enzyme activity corresponds to the amount of enzyme that catalyzes the reduction of one nM of NADPH per minute.

3.4.6. Estimation of Glutamate Synthase

Glutamate synthase activity was assayed as per the procedure described by Singh *et al.* (1983). One gram of leaf sample was ground in five ml of 0.1 M potassium phosphate buffer (pH 7.5) (Appendix V) containing one mM EDTA, one mM dithioerythritol and a pinch of PVP. The homogenate was centrifuged at 10,000 rpm for 30 minutes at 4^0C and the supernatant was used as crude enzyme for the assay. Four ml reaction mixture contained one ml glutamine, one ml α- ketoglutarate, one ml NADPH, 1.8 ml phosphate buffer and 0.2 ml enzyme extract. The blank used contained all the components of reaction mixture but α-ketoglutarate. The reaction mixture was incubated at 37^0C for 30 minutes, and the change in absorbance at 340 nm was recorded.

3.4.7. Estimation of Phosphatase

Phosphatase activity was estimated as per the procedure described by Lowry *et al.* (1954). One gram of leaf tissue was homogenized in 10 ml of ice-cold 0.05 M sodium citrate buffer (pH 5.3) (Appendix V), in a chilled pestle and mortar. The homogenate was centrifuged at 12,000 rpm for 10 minutes and the supernatant was used as crude enzyme for the assay. 0.5 ml of the enzyme extract was added to three ml of substrate solution (Appendix V), incubated at 37^0C for five minutes and was mixed well. 0.5 ml was removed immediately from this mixture and was added to 9.5 ml of 0.085 N sodium hydroxide (NaOH) which served as the blank. The remaining solution was incubated at 37^0C for 15 minutes and 0.5 ml of this mixture was taken and added to 9.5 ml of 0.085 N NaOH. The absorbance of the solution was read at 405 nm, against the blank. The standard curve for phosphatase was prepared using *p*- nitrophenol and the phosphatase activity in the sample was expressed as mM of *p*- nitrophenol produced per minute per g of tissue.

3.4.8. Estimation of Superoxide Dismutase (SOD)

SOD activity was estimated as per the method described by Dhindsa *et al.* (1981), in which SOD activity was determined by measuring the inhibition in photo-reduction of NBT by the enzyme SOD. For this, one gram of leaf tissue was homogenized in 10 ml of ice-cold 0.05 M potassium phosphate buffer (pH 7.8) and a pinch of PVP, in a chilled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 10 minutes at 4^0C, and the supernatant was used as enzyme source for the assay. Reaction mixture (three ml) was prepared using 13 mM L-methionine, two mM riboflavin, 75 µM nitroblue tetrazolium chloride (NBT), 0.1 mM ethylenediamine tetraacetic acid (EDTA) and 50 µl enzyme extract, taken in tubes. Ribloflavin was added at last, and the tubes were shaken and exposed to white light for 15 minutes at room temperature. Reaction mix containing all other components but enzyme extract, was used as blank, and the absorbance at 560 nm was recorded. The per cent inhibition in photo-reduction of NBT by SOD was calculated as:

$$\text{Per cent inhibition} = \frac{A_{560} \text{ of control} - A_{560} \text{ of sample}}{A_{560} \text{ of control}} \times 100$$

NBT undergoes photo-reduction on exposure to light by superoxide radicals. It competes with SOD for superoxide anions. In the presence of SOD, NBT will produce lesser amount of coloured complex than control. One unit of SOD activity corresponds to the amount of enzyme required for causing 50 per cent inhibition of photo-reduction of NBT.

3.4.9. Detection of Superoxides (O_2^-) by Nitro Blue Tetrazolium Chloride (NBT) Staining and Hydrogen Peroxide (H_2O_2) by Diaminobenzidine (DAB) Staining

The staining technique was performed as per the procedure described by Kumar *et al*. (2014). The leaf samples were thoroughly washed in distilled water to remove the impurities, if any. They were placed in Petri-dishes and immersed in NBT or DAB staining solution (Appendix VII) for the detection of O_2^- or H_2O_2 respectively. The plates were wrapped in aluminium foil and kept overnight in a shaker, at room temperature. The excess stain was drained off from the plates, and the leaves were immersed in absolute alcohol and kept in a boiling water bath with intermittent shaking, until the chlorophyll was completely removed. The leaves were then transferred to paper towels saturated with 60 per cent glycerol and the stained leaves were photographed.

Results

4. RESULTS

The research work entitled "Evaluation of *Piriformospora indica* for the management of *Piper yellow mottle virus* in black pepper" was carried out in the Department of Plant Pathology, College of Agriculture, Vellayani during the period 2019-2021, to evaluate the beneficial fungal root endophyte *P. indica* for the management of PYMoV in black pepper and to elucidate the role of reactive oxygen species and antioxidant enzymes in the tripartite interaction. The results of the study are detailed in this chapter.

4.1. INCIDENCE AND SEVERITY OF PYMoD IN BLACK PEPPER

All the surveyed black pepper varieties were severely infected with PYMoV, with disease incidence varying from 10 to 91 per cent (Table 5). Among the varieties surveyed, var. Karimunda had the highest D. I. of 91 per cent, followed by Panniyur 6 (D.I - 71 per cent) and Panniyur 1 (D. I. - 49 per cent). Var. Karimunda was found to be highly susceptible to PYMoV, with disease severity assessed as V. I. of 58, Panniyur 6 (V. I.- 45) was found to be susceptible, Panniyur 1 (V. I.- 37), Panniyur 2 (V. I.- 30), Panniyur 3 (V. I.- 33), Panniyur 4 (V. I.- 33), Panniyur 5 (V. I.- 34) and Panniyur 7 (V. I.- 34) were found to be moderately susceptible to PYMoV infection (Table 6).

The characteristic symptoms of PYMoV in different black pepper varieties were studied (Plate 4, Plate 5 and Plate 6). Symptoms were observed mostly on the leaves. All the varieties exhibited almost similar symptoms, the severity of which varied from variety to variety. The most severe symptoms were shown by var. Karimunda and Panniyur 1.

PYMoV-infected plants initially showed chlorotic specks and flecks, which later developed into mottles. The mottling symptoms become clearly visible when held against light. The chlorotic mottles on advancement were found to develop necrotic centres. Necrotization was more prominent in the variety Panniyur 1. Crinkling and distortion of leaves and reduction in leaf size were also observed. In severe cases, stunting of the plants was observed, mostly in *var.* Karimunda and Panniyur 1, due to reduction in the internodal length.

4.2. MOLECULAR DETECTION OF PYMoV

PCR Amplification

The total genomic DNA was isolated from the collected samples by CTAB method. Good quality DNA samples that showed A_{260} / A_{280}values around 1.8 were subjected to PCR using primer pair specific to ORF III of PYMoV (Table 2) as per the conditions specified (Table 4). All the samples yielded PCR products of amplicon size 400 bp; thus confirming the presence of PYMoV in the collected samples (Plate 7 and Plate 8).

4.3. EVALUATION OF *P. indica*-COLONIZED BLACK PEPPER CUTTINGS AGAINST PYMoV

4.3.1. Maintenance of *P. indica*

P. indica obtained from Department of Plant Pathology was maintained in Petri-dishes containing potato dextrose agar (PDA). It was sub-cultured at fortnightly intervals to prevent loss of virulence. Radial growth of the fungal mycelium fully covered the nine cm Petri-dish at 25 days after inoculation (DAI). In potato dextrose broth (PDB), full growth of the fungus was obtained at 15 DAI. In the mass-multiplication medium (1:1 coirpith-dried farmyard manure mixture amended with two per cent gram flour), optimum growth of the fungus was obtained at seven DAI (Plate 9).

Table 5: Incidence of PYMoD in different black pepper varieties

Sl. No.	Variety	D. I. (%)*
1	Panniyur 1	49
2	Panniyur 2	24
3	Panniyur 3	15
4	Panniyur 4	10
5	Panniyur 5	12
6	Panniyur 6	71
7	Panniyur 7	47
8	Karimunda	91

*Total number of plants surveyed under each variety = 100

Table 6: Categorization or disease severity assessed as vulnerability index of different black pepper varieties to PYMoV in the survey

Variety	Vulnerability index*	Category
Panniyur 1	37	Moderately susceptible
Panniyur 2	30	Moderately susceptible
Panniyur 3	33	Moderately susceptible
Panniyur 4	33	Moderately susceptible
Panniyur 5	34	Moderately susceptible
Panniyur 6	45	Susceptible
Panniyur 7	34	Moderately susceptible
Karimunda	58	Highly susceptible

*Total number of plants surveyed under each variety = 25

4.3.2. Co-cultivation of Rooted Cuttings of Black pepper with *P. indica*

Rooted cuttings of black pepper var. Panniyur 1 were planted in polybags containing *P. indica*-mass multiplied medium, after surface sterilizing the roots. The roots were assessed for *P. indica*-colonization every other day, by trypan blue staining. Microscopic examination showed the presence of pear-shaped chlamydospores of *P. indica* on the surface of black pepper roots seven days after co-cultivation (DAC). Chlamydospores were seen inside the root cortex 15 DAC (Plate 10).

4.3.3. Effect of *P. indica*- Colonization on Growth of Black pepper Plants

Effect of *P. indica*-colonization on different biometric parameters of black pepper plants were assessed at monthly intervals after co-cultivation. *P. indica*-colonization resulted in significant increase in shoot length, root length, number of leaves, leaf area and number of primary roots compared to the control plants. At 30 DAC, *P. indica*-colonized plants showed 11 per cent more shoot length, 24 per cent more root length, 20 per cent more number of leaves, 15 per cent more leaf area and 27 per cent more number of primary roots than the control plants (Table 7, 8, 9, 10 and 11). The difference in most of the growth parameters between the treated and control plants was more pronounced at 60 DAC, wherein the colonized plants showed 24 per cent more shoot length, 26 per cent more root length, 46 per cent more number of leaves, 32 per cent more leaf area and 33 per cent more number of primary roots (Table 7, 8, 9, 10 and 11). After this, the difference in growth was only gradual. *P. indica*-colonized plants showed 25 per cent more shoot length and 23 per cent more root length, 35 per cent more number of leaves, 23 per cent more leaf area and 27 per cent more number of primary roots than the control plants, at 150 DAC (Table 7, 8, 9, 10 and 11).

4.3.4. Evaluation of *P. indica*-Colonized Black pepper Cuttings against PYMoV

A pot culture experiment was laid in CRD in Department of Plant Pathology, to evaluate the efficiency of prophylactic as well as curative application *P. indica* in managing PYMoV in black pepper var. Panniyur 1. For evaluating the effect of prophylactic application of *P. indica* in managing PYMoV infection, the virus infected scions were grafted onto *P. indica*-colonized rooted cuttings of var. Panniyur 1 at zero, two, seven, 15 and 30 DAC; and for the curative treatment, *P. indica* was made to colonize on rooted cuttings that were already infected with PYMoV.

PCR amplification of the DNA isolated from the first emerged leaves of the post-inoculation treatments (T4, T5, T6, T7 and T8) revealed T8 (*P. indica*-colonization on healthy rooted cuttings, followed by grafting PYMoV-infected scion after 30 days) as the best post-inoculation treatment, as it showed the least virus titer (Plate 12 and 13). Thus T8 alone was taken hereafter as the post-inoculation treatment for the further study.

Days taken for symptom development, disease incidence and vulnerability index were calculated at 30, 60 and 90 days after treatments. Post- inoculation of PYMoV on *P. indica*-colonized plants could delay the symptom expression of PYMoV, as compared to the un-colonized plants, upon artificial inoculation of the virus (Table 12).

P. indica-colonization in black pepper cuttings could reduce the incidence of PYMoD when the virus was post-inoculated at 15 and 30 DAC (T7 and T8). Though the effect of *P. indica*-colonization was not much obvious in lowering the incidence of PYMoD, it could produce a pronounced effect in reducing the severity of PYMoD, as evident from the vulnerability index. *P. indica* colonization followed by post-inoculation of PYMoV after 30 days (T8) resulted in the lowest V. I. (28.75); which was 65 per cent lesser than the V. I. of the non-colonized diseased plants (82.14), when observed at 90 days after treatments. *P. indica*-colonization on pre-inoculated plants (T3) resulted in 34.8 per cent decrease in V. I. compared to the diseased control plants (T2) (Table 13). Thus, though both prophylactic as

well as curative treatment with *P. indica* could lower the V. I. of black pepper to PYMoV, prophylactic treatment offered better results.

P. indica-colonized plants that were post-inoculated with the virus (T8) showed only mild symptoms like chlorotic mottling and slight leaf crinkling, even at 90 days after inoculation with the virus. *P. indica*-colonization on plants pre-inoculated with PYMoV (T3) did not result in any symptom remission in the already infected leaves; but the newly emerged leaves showed only milder symptoms compared to the already infected ones. However, the un-colonized control plants inoculated with the virus alone (T2) showed severe symptoms like crinkling and distortion of leaves, drastic reduction in leaf area and stunted growth.

Biometric parameters like plant height, number of leaves and leaf area were significantly higher (at five per cent level of significance) in *P. indica*-colonized plants upon pre- as well as post-inoculation of PYMoV (T3 and T8) compared to the diseased control plants (T2). *P. indica*-colonized plants that were post-inoculated with the virus (T8) showed 31 per cent more shoot length, 60 per cent more number of leaves and 65 per cent more leaf area than the non-colonized diseased plants (T2), at 90 days after treatments. Similarly, plants pre-inoculated with the virus and then colonized by *P. indica* (T3) showed 24 per cent more shoot length, 50 per cent more number of leaves and 45 per cent more leaf area than the non-colonized control plants (T2), on graft inoculation of PYMoV (Table 14). Surprisingly, the number of leaves and leaf area of the post-inoculation treatment was comparable to that of the absolute control plants. Number of branches was more in the post-inoculation treatment compared to the control plants, which may possibly be due to the breakage of apical dominance, due to grafting.

Amplification by PCR using the virus specific primers also revealed that virus titer was the least in *P. indica*-colonized plants that were post-inoculated with the virus, followed by pre-inoculated plants that were later colonized with *P. indica;* and was the highest in the un-colonized plants inoculated with the virus.

Plate 3: Maintenance of PYMoV-infected black pepper samples under insect-proof condition

Chlorotic mottling | **Crinkling and distortion of leaves** | **Distortion and reduction of leaf size** | **Stunted growth**

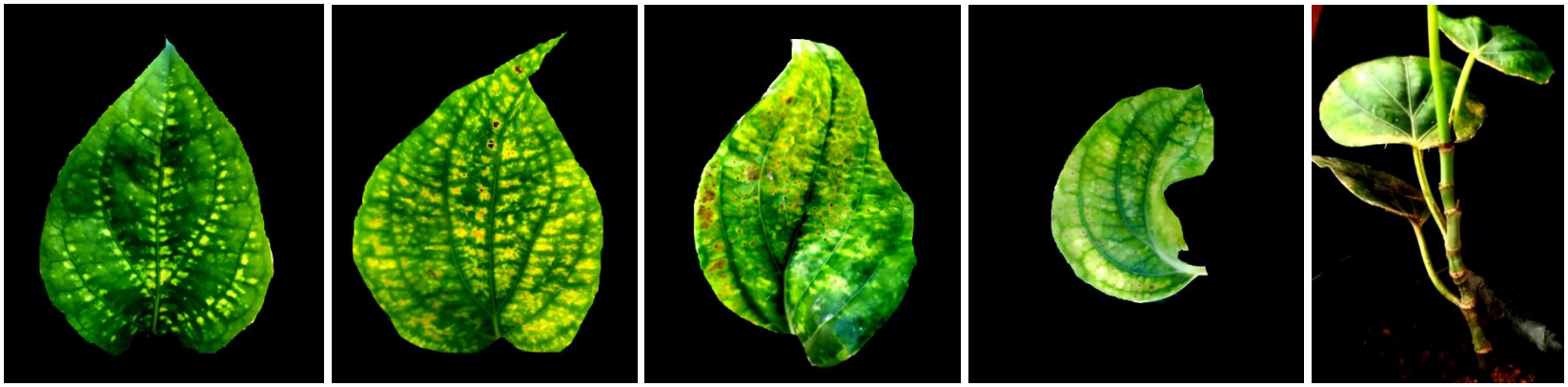
Plate 4: Symptoms of PYMoV in *var*. Karimunda

Chlorotic mottling

Crinkling and distortion of leaves

Reduction in leaf size

Plate 5: Symptoms of PYMoV in variety Panniyur 6



Chlorotic mottling | **Chlorotic mottling with necrotic spots** | **Crinkling and distortion of leaves** | **Reduction in leaf size** | **Stunted growth**

Plate 6: Symptoms of PYMoV in variety Panniyur 1

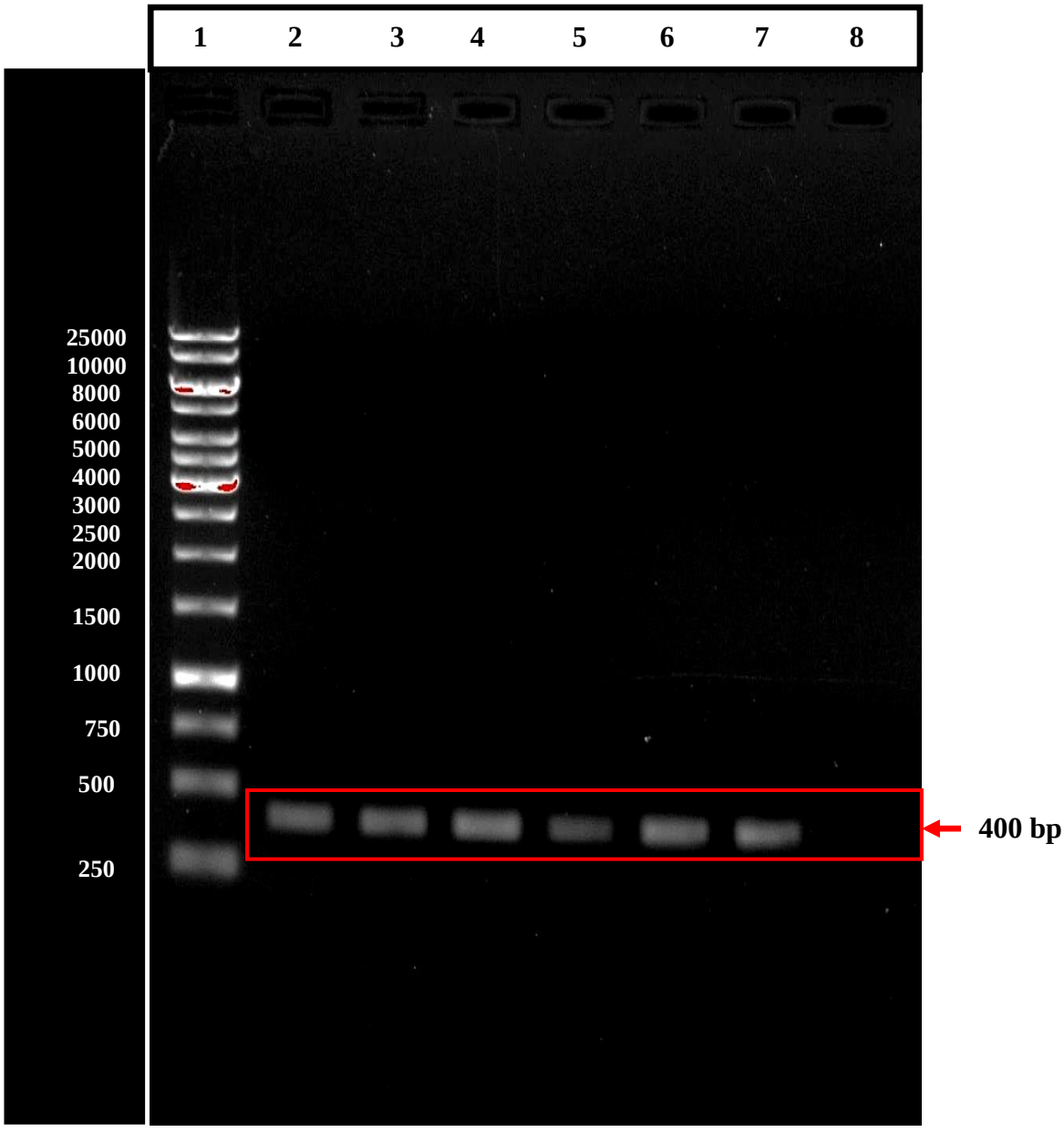


Plate 7: Electrophoresis gel image of amplified DNA of PYMoV using primer pair specific to ORF III: Lane (1) 1 kb marker (2) Panniyur 1 (3) Panniyur 2 (4) Panniyur 3 (5) Panniyur 4 (6) Panniyur 5 (7) Panniyur (8) Negative control

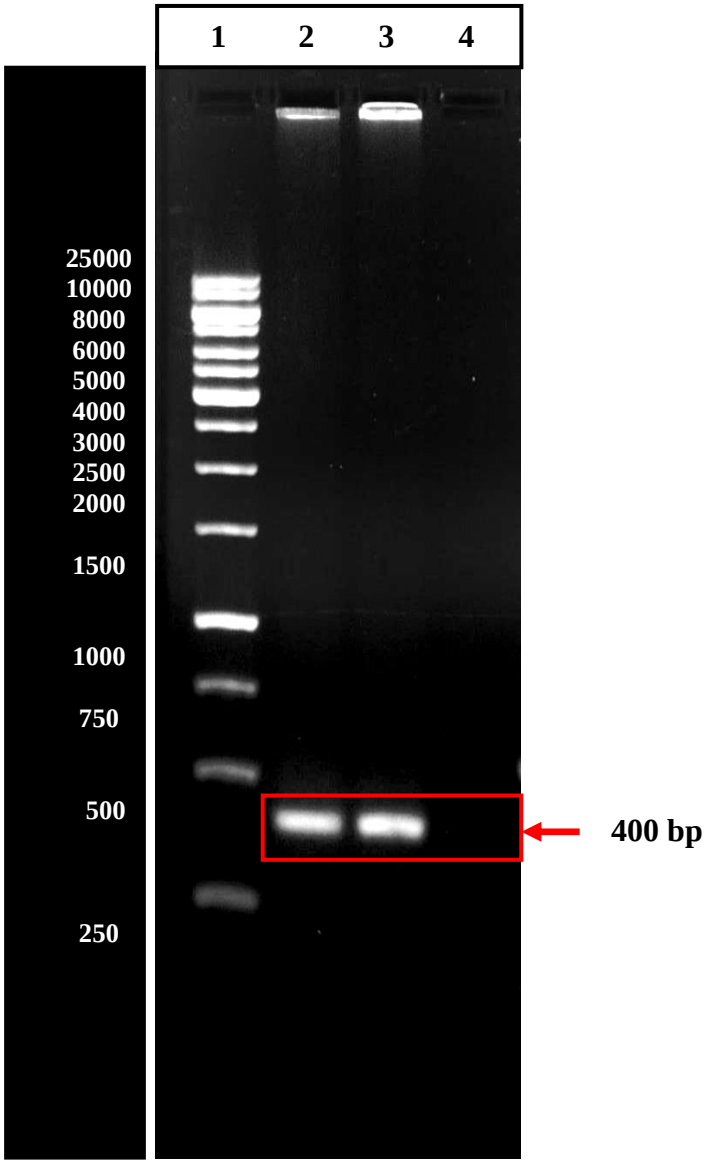


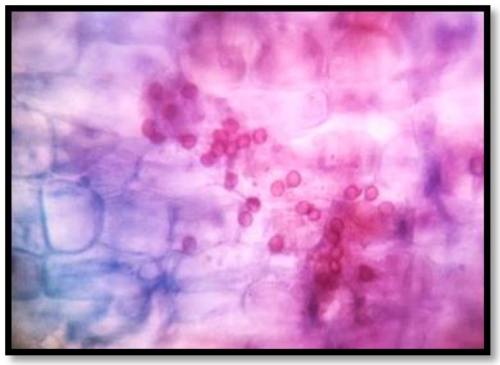
Plate 8: Electrophoresis gel image of amplified DNA of PYMoV using primer pair specific to ORF III: Lane (1) 1 kb marker (2) Panniyur 7 (3) Karimunda (4) Negative control

(a)

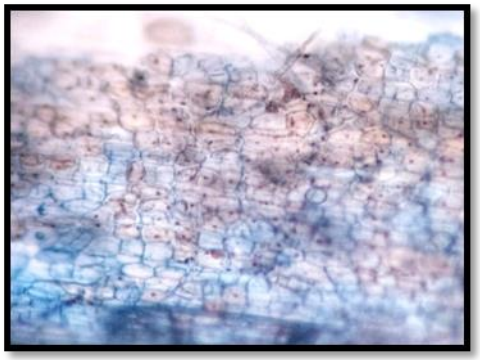
(b)

(c)

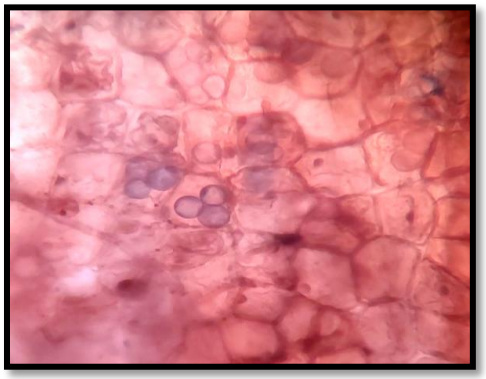
Plate 9: Maintenance of *P. indica* (a) Growth of *P. indica* in PDA- 25 days after inoculation (b) Growth of *P. indica* in PDB- 15 days after inoculation (c) Growth of *P. indica* in coirpith- dried FYM (1:1) mixture amended with 2 per cent gram flour- 5 days after inoculation



(a)



(b)



(c)

Plate 10: Chlamydospores of *P. indica*: (a) on the surface of roots at 7 days after co-cultivation (b) and (c) inside the root cortex at 25 days after co-cultivation

(a) **(b)**

Plate 11: Effect of *P. indica* colonization on different biometric parameters of black pepper at (a) 30 days after co-cultivation (b) 90 days after co-cultivation

Table 7: Effect of *P. indica*-colonization on shoot length of black pepper var. Panniyur 1

Treatments	Shoot length (cm)				
	Before co-cultivation	**30 DAC**	**60 DAC**	**90 DAC**	**150 DAC**
+ *P. indica*	18.26 ± 0.799	26.20 ± 1.764	42.96 ± 1.660	68.02 ± 1.446	125.18 ± 3.241
- *P. indica*	18.54 ± 0.650	23.32 ± 0.638	32.70 ± 1.014	54.92 ± 0.785	93.06 ± 4.098
T- calculated	-	3.43	11.79	17.20	13.75
T (0.05)	2.306				

DAC- Days after co-cultivation; Values are the means of five replications ± Standard deviation

Table 8: Effect of *P. indica*-colonization on root length of black pepper var. Panniyur 1

Treatments	Root length (cm)				
	Before co-cultivation	**30 DAC**	**60 DAC**	**90 DAC**	**150 DAC**
+ *P. indica*	9.50 ± 0.616	14.98 ± 0.531	21.12 ± 0.402	28.20 ± 0.731	36.30 ± 0.587
- *P. indica*	9.34 ± 0.673	11.42 ± 0.746	15.70 ± 0.543	21.46 ± 0.698	27.82 ± 0.507
T- calculated	-	8.691	17.928	14.901	24.439
T (0.05)	2.306				

DAC- Days after co-cultivation; Values are the means of five replications ± Standard deviation

Table 9: Effect of *P. indica*-colonization on number of leaves in black pepper var. Panniyur 1

Treatments	Number of leaves				
	Before co-cultivation	**30 DAC**	**60 DAC**	**90 DAC**	**150 DAC**
+*P. indica*	2 ± 0.548	5 ± 0.707	13 ± 1.140	22 ± 3.847	34 ± 6.041
- *P. indica*	2 ± 0.548	4 ± 0707	8 ± 1.140	15 ± 2.408	22 ± 4.427
T-calculated	-	2.236	5.270	3.843	4.427
T (0.05)	2.306				

DAC- Days after co-cultivation; Values are the means of five replications ± Standard deviation

Table 10: Effect of *P. indica*-colonization on leaf area of black pepper var. Panniyur 1

Treatments	Leaf area (cm^2)				
	Before co-cultivation	**30 DAC**	**60 DAC**	**90 DAC**	**150 DAC**
+*P. indica*	35.4 ± 3.401	47.1 ± 3.238	70.15 ± 3.945	81.95 ± 5.146	86.20 ± 7.502
- *P. indica*	36.8 ± 2.419	39.85 ± 2.742	47.90 ± 4.509	58.95 ± 5.538	66.05 ± 6.426
T-calculated	-	3.569	7.888	6.440	4.264
T (0.05)	2.306				

DAC- Days after co-cultivation; Values are the means of five replications ± Standard deviation

Table 11: Effect of *P. indica*-colonization on number of primary roots in black pepper var. Panniyur 1

Treatments	**Number of primary roots**				
	Before co-cultivation	**30 DAC**	**60 DAC**	**90 DAC**	**150 DAC**
+*P. indica*	5 ± 0.707	11 ± 0.707	18 ± 1.140	24 ± 2.121	37 ± 1.516
- *P. indica*	5 ± 0.707	8 ± 1.140	12 ± 1.483	18 ± 1.341	27 ± 1.673
T-calculated	-	4.333	6.932	5.880	9.901
T (0.05)	2.306				

DAC- Days after co-cultivation; Values are the means of five replications ± Standard deviation

Table 12: Effect of *P. indica*-colonization on PYMoD symptom appearance in black pepper plants (var. Panniyur 1)

Treatments	Days taken for symptom appearance
T1 (*P. indica* alone)	-
T2 (PYMoV alone)	29
T3 (PYMoV + *P. indica*)	-
T4 (*P. indica*+ PYMoV at zero DAC)	42
T5 (*P. indica*+ PYMoV at two DAC)	37
T6 (*P. indica*+ PYMoV at seven DAC)	41
T7 (*P. indica*+ PYMoV at 15 DAC)	50
T8 (*P. indica*+ PYMoV at 30 DAC)	56
T9 (Absolute control)	-

*****Number of plants observed under each treatment = 7

Table 13: Effect of *P. indica*-colonization on incidence and severity of PYMoD in black pepper var. Panniyur 1

Treatments	Disease Incidence (%)			Vulnerability Index		
	30 DAT	60 DAT	90 DAT	30 DAT	60 DAT	90 DAT
T1 (*P. indica*alone)	-	-	-	-	-	-
T2 (PYMoV alone)	85.71	100	100	42.86	71.42	82.14
T3 (PYMoV + *P. indica*)	100	100	100	39.28	50.00	53.57
T4 (*P. indica*+ PYMoV at zero DAC)	42.85	100	100	25.00	35.71	38.10
T5 (*P. indica*+ PYMoV at two DAC)	28.57	85.71	100	14.28	39.28	42.86
T6 (*P. indica*+ PYMoV at seven DAC)	42.85	71.43	100	21.43	28.57	35.71
T7 (*P. indica*+ PYMoV at 15 DAC)	28.57	71.43	71.43	14.28	28.57	32.14
T8 (*P. indica*+ PYMoV at 30 DAC)	14.28	57.14	71.43	7.14	25.00	28.57
T9 (Absolute control)	-	-	-	-	-	-

*Number of plants observed for disease incidence and vulnerability index under each treatment= 7

Table 14: Effect of *P. indica*-colonization in black pepper plants (var. Panniyur 1) against PYMoV upon graft transmission; at 90 days after treatments

Treatments	Plant height (cm)	Number of branches	Number of leaves	Leaf area (cm^2)
T1 (*P. indica* alone)	69.62 ± 5.327 [a]	3 ± 0.707 [a]	22 ± 4.899 [a]	81.15 ± 5.851 [a]
T2 (PYMoV alone)	28.82 ± 3.716 [d]	2 ± 0.548 [b]	6 ± 1.483 [c]	16.00 ± 4.377 [d]
T3 (PYMoV + *P. indica*)	38.72 ± 4.499 [c]	2 ± 0.707 [b]	15 ± 4.393 [b]	28.90 ± 5.784 [c]
T8 (*P. indica* + PYMoV)	41.97 ± 4.393 [c]	3 ± 0.707 [a]	12 ± 2.191 [b]	45.65 ± 9.015 [b]
T9 (Absolute control)	56.23 ± 5.800 [b]	2 ± 0.447 [b]	15 ± 2.236 [b]	55.25 ± 10.798 [b]
SE (m) ±	2.123	0.297	1.359	3.204
CD (0.05)	6.337	0.834	4.388	9.955

Values are the means of five replications ± standard deviation; SE: Standard error; CD: Critical difference;

Superscripts with same alphabets indicate on par values and those in different alphabets indicate significant difference at 5 per cent level of significance.

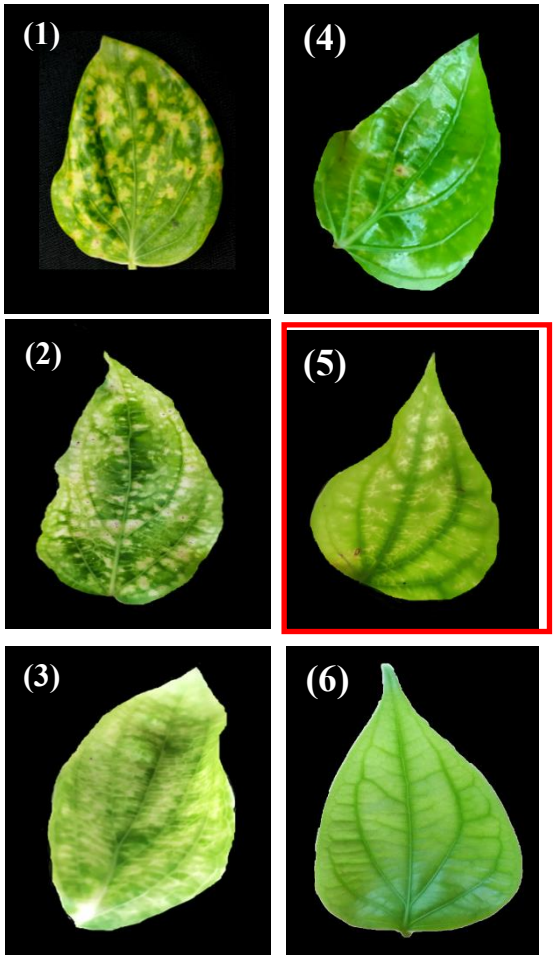


Plate 12: Symptoms of PYMoD on 1st emerged leaf of post-inoculation treatments: (1) T4 (2) T5 (3) T6 (4) T7 (5) T8 (6) T9

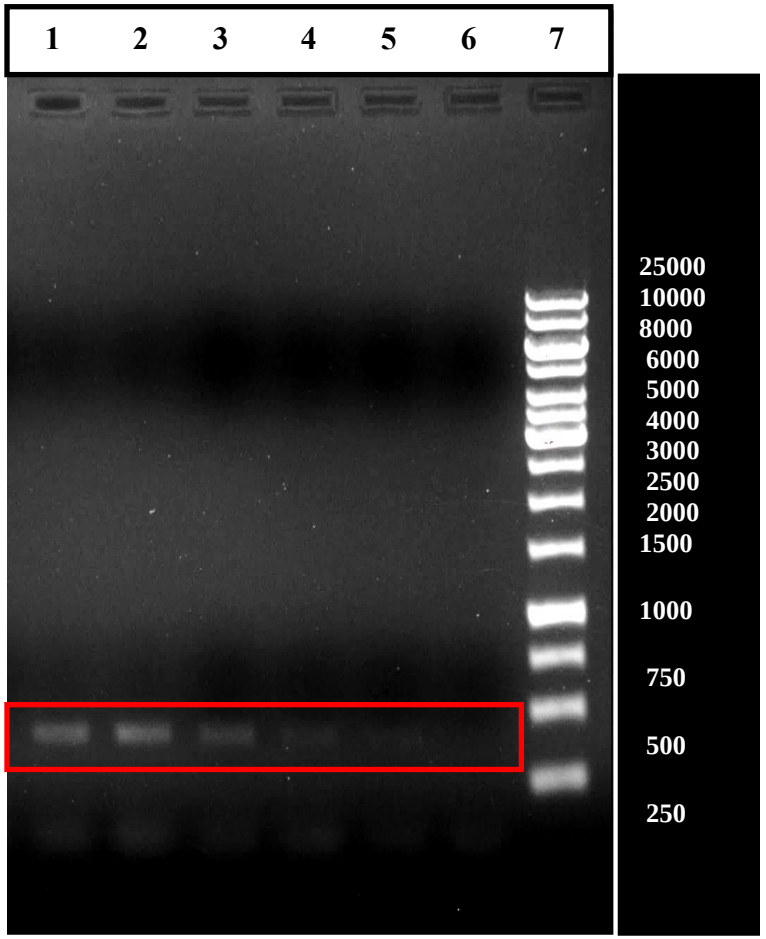


Plate 13: PCR amplification of the 1st emerged leaf of the post-inoculation treatments, using PYMoV specific primers: Lanes (1) T4 (2) T5 (3) T6 (4) T7 (5) T8 (6) Negative control (7) 1 kb ladder

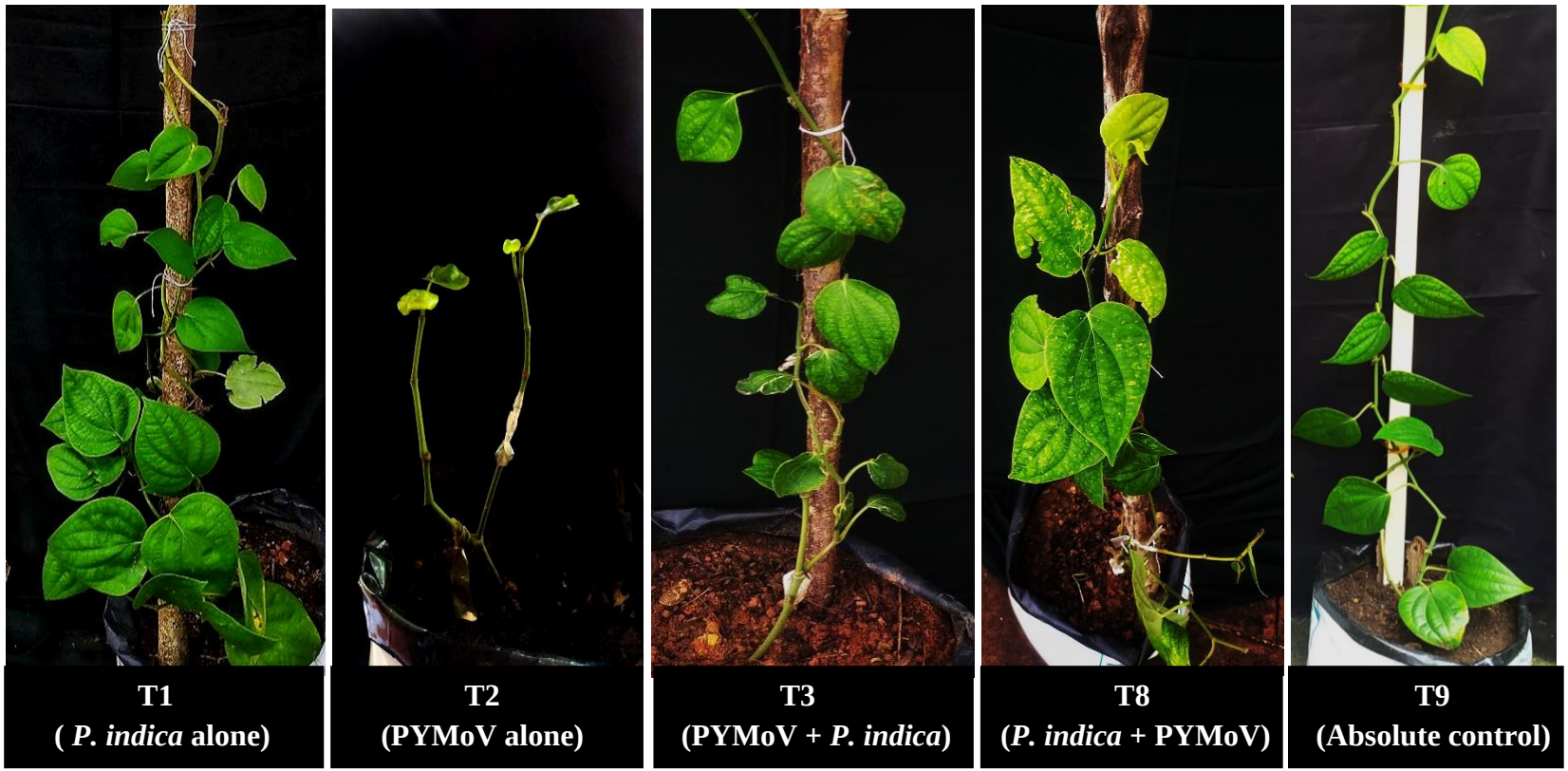


Plate 14: Effect of *P. indica*-colonization in black pepper plants against PYMoV on graft transmission at 90 days after treatment

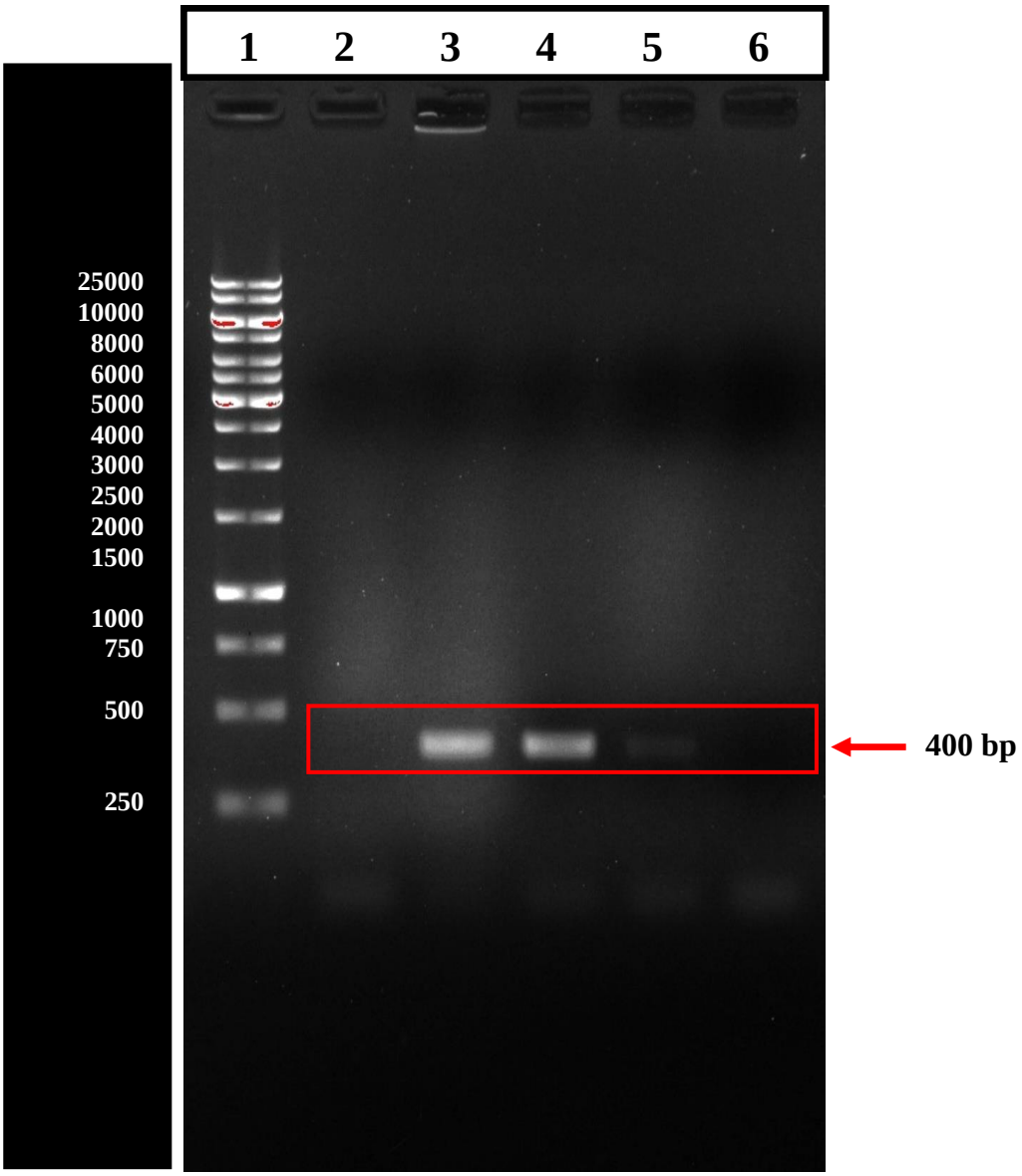


Plate 15: Electrophoresis gel image of amplified DNA of PYMoV using primer pair specific to ORF III of PYMoV. Lane (1) 1 kb ladder (2) T1 (*P. indica* alone) (3) PYMoV alone (4) T3 (PYMoV + *P. indica*) (5) T8 (*P. indica* + PYMoV) (6) Absolute control

4.4. ELUCIDATING THE ROLE OF ROS AND ANTIOXIDANT ENZYMES IN THE TRIPARTITE INTERACTION

In order to evaluate the efficiency of *P. indica* in modulating the biochemical responses of black pepper plants to PYMoV infection, the activity of various enzymes like catalase, peroxidase, glutathione reductase, glutamate synthase, phosphatase and superoxide dismutase, which are known to play important roles in the antioxidant defense system of plants were assayed at 15, 45, 60 and 90 days after the treatments. Also, the presence of superoxides was detected using nitroblue tetrazolium chloride (NBT) staining and hydrogen peroxide was detected using diaminobenzidine (DAB) staining.

4.4.1. Chlorophyll Content

Chlorophyll content was estimated as per the procedure described by Arnon (1949). Throughout the study, PYMoV infection significantly reduced the chlorophyll content of the infected plants (T2) compared to the healthy plants (T9). *P. indica* colonized black pepper plants had 27.8 per cent, 28.6 per cent, 25 per cent and 15.8 per cent more chlorophyll content than the non- colonized plants at 15, 45, 60 and 90 DAT respectively.

In the non-colonized plants challenged by the virus, the total chlorophyll content was deteriorating throughout the intervals studied. Whereas, in the *P. indica* colonized plants inoculated with the virus (pre- as well as post-), the chlorophyll content was getting better throughout the study, though not up to the level of the absolute control plants; indicating that *P. indica* is helping the plants to overcome the loss of chlorophyll caused due to PYMoV infection.

P. indica-colonized plants that were post-inoculated with PYMoV (T8) showed significantly higher total chlorophyll content, compared to the non-colonized diseased plants (T2) at all the intervals studied. Chlorophyll content of T8 was 24.1 per cent, 27.8 per cent, 58 per cent and 66.3 per cent higher than T2 at 15, 45, 60 and 90 DAT. Colonization of *P. indica* in plants pre-inoculated with PYMoV (T3), also showed an

increase in total chlorophyll content over the virus inoculated control plants (T2). This increase in chlorophyll content, though not significant initially (at 15, 45 and 60 days after treatments), showed a significant increase later (at 90 days after treatments), which was on par with the post-inoculation treatment. T3 had 1.5 per cent, 12.5 per cent, 37.8 per cent and 54.6 per cent more chlorophyll content than T2 at 15, 45, 60 and 90 DAT (Table 15) (Fig. 3).

4.4.2. Total Protein Content

Total soluble protein content was estimated as per the protocol of Bradford (1976). *P. indica*-colonization (T1) significantly increased the total soluble protein content compared to the absolute control plants (T9). PYMoV infection in the non-colonized (T2) plants also increased the total protein content compared to the absolute control plants (T9).

Among the virus-infected plants, total soluble protein content was the highest in *P. indica*-colonized plants that were post- inoculated with PYMoV (T8), at all the intervals studied. In the non- colonized plants inoculated with PYMoV, the total protein content at 15 and 45 DAT were significantly higher than in the absolute control plants, but at later stages the content got reduced to a level that was on par with the absolute control plants. Colonization of *P. indica* on plants pre-inoculated with PYMoV (T3) initially showed protein contents that were on par with the un-colonized diseased plants (T2), but at later stages (at 60 and 90 days after treatments) showed values that were significantly higher than the latter, although not as high as the post- inoculation treatment (T8) (Table 16) (Fig. 4).

4.4.3. Catalase Activity

Catalase activity was assayed calorimetrically as per the procedure described by Luck (1974). Catalase activity was the highest in *P. indica*-colonized healthy plants (T1) and was around five-fold higher than the un-colonized healthy plants (T9), at all the

intervals studied. PYMoV infection significantly increased the catalase activity compared to the healthy plants, throughout the study.

Both pre- as well as post-inoculation of PYMoV on *P. indica*-colonized plants (T3 and T8) resulted in a significant increase in catalase activity over the un-colonized diseased plants (T2). Catalase activity in the pre- and post-inoculated plants were initially on par, but at later stages, post-inoculated plants (T8) showed better activity than the latter (T3) (Table 17). In the colonized plants challenged by the virus, the highest catalase activity was recorded at 15 DAT, and thereafter the activity was found to be decreasing, though it was higher compared to the un-colonized plants (Table 17) (Fig. 5).

4.4.4. Peroxidase Activity

Peroxidase activity was assayed as per the protocol of Srivastava (1987). *P. indica*-colonization in healthy plants (T1) resulted in almost two fold increase in peroxidase activity over the absolute control plants (T9). PYMoV infection resulted in significant increase in peroxidase activity compared to healthy plants.

Among the virus-infected plants, the highest peroxidase activity was recorded in *P. indica*-colonized plants that were post-inoculated with PYMoV (T8), which was comparable with the *P. indica*-colonized healthy plants (T1). *P. indica* colonization on plants pre- inoculated with PYMoV (T3) also showed significantly higher peroxidase activity than the un-colonized diseased plants (T2), but was not as high as the post-inoculated plants (T8) (Table 18) (Fig. 6).

4.4.5. Glutathione Reductase Activity

Glutathione reductase (GR) activity was estimated as per the protocol of Smith *et al.* (1988). There was a significant increase in GR activity in *P. indica*-colonized healthy plants (T1) when compared to non-colonized control plants (T9). Also, virus infection significantly increased the GR activity compared to the healthy plants.

Pre- as well as post-inoculation of PYMoV on *P. indica*-colonized plants (T3 and T8) resulted in significantly higher glutathione reductase activity compared to the un-colonized diseased plants (T2), at all the intervals studied. *P. indica*-colonized plants that were post-inoculated with PYMoV (T8) showed the highest GR activity among the virus-infected plants, which was on par with the *P. indica*-colonized healthy plants (T1). In the pre-inoculation treatment, the GR activity showed an increasing trend till 60 DAT, but at 90 DAT there was a drop in GR activity; whereas in the post-inoculation treatment an increasing trend was observed throughout the study (Table 19) (Fig. 7).

4.4.6. Glutamate Synthase Activity

Glutamate synthase activity was assayed as per the procedure described by Smith *et al.* (1983). Significant increase in glutamate synthase activity was observed in *P. indica*-colonized healthy plants (T1) compared to the un-colonized control plants (T9).

However, pre- as well as post-inoculation of the virus on *P. indica*-colonized plants (T3 and T8) did not result in any significant increase in glutamate synthase activity when compared to the un-colonized plants challenged by the virus (T2) (Table 20) (Fig. 8).

4.4.7. Phosphatase Activity

Phosphatase activity was assayed as per the protocol of Lowry *et al.* (1954). Activity of phosphatase enzyme was significantly higher in *P. indica*-colonized healthy plants (T1) than in the absolute control plants (T8). PYMoV infection significantly increased phosphatase activity at all the intervals observed, compared to the absolute control plants.

Pre- as well as post-inoculation of the virus on *P. indica*-colonized plants (T3 and T8) resulted in significant increase in phosphatase activity when compared to the non-colonized diseased plants (T2) at all the intervals studied (Table 21) (Fig. 9).

4.4.8. Superoxide Dismutase Activity

SOD activity was assayed as per the procedure described by Dhindsa (1981), by measuring the inhibition in photo-reduction of nitrobluetetrazolium (NBT) by the enzyme SOD. *P. indica*-colonized healthy plants (T1) showed significantly higher SOD activity compared to the un-colonized control plants (T9). PYMoV infection resulted in a significant increase in SOD activity compared to the control plants at all the intervals studied.

Among the virus-infected plants, *P. indica*-colonization followed by post-inoculation of PYMoV (T8) resulted in the highest SOD activity. SOD activity in *P. indica*-colonized plants pre-inoculated with PYMoV (T3) was also found to be significantly higher than the non- colonized diseased plants (T2). Higher SOD activity was recorded at 15 DAT itself, but at 45 DAT there was an abrupt hike in SOD activity in the colonized as well as the un-colonized plants infected with the virus. Thereafter the activity recorded a decreasing trend in the un-colonized plants; but the colonized plants still exhibited an increasing trend (Table 22) (Fig. 10).

4.4.9. Detection of Superoxides (O_2^-) and Hydrogen Peroxide (H_2O_2) in Leaves of *P. indica*-Colonized Black pepper Plants upon Pre- and Post- Inoculation of PYMoV

Nitrobluetetrazolium chloride (NBT) and diaminobenzidine (DAB) were used as chromogens for the histochemical detection of superoxide anions and hydrogen peroxide respectively, in black pepper leaves.

NBT specifically reacts with superoxide anions and form a blue colour precipitate of formazan. O_2^- accumulation was almost nil in the *P. indica*-colonized healthy plants as well as the absolute control plants. A steady increase in O_2^- accumulation was observed in the non-colonized plants challenged by the virus alone, with the highest O_2^- accumulation at 90 DAT. *P. indica*-colonization resulted in a significant reduction in this effect upon pre- as well as post-inoculation of PYMoV. At 90 DAT, the O_2^-

accumulation was found to be negligible in the colonized plants compared to the un-colonized plants challenged by the virus (Plate 16, 18, 20 and 22).

DAB gets oxidized in the presence of proteins like peroxidases, and generate a brown-colour precipitate. This precipitate serves as a stain to detect the presence and distribution of hydrogen peroxide in the plant tissue. From the present study, it was observed that both the *P. indica*-colonized healthy plants and the absolute control plants had essentially no H_2O_2 buildup. The non-colonized plants challenged by the virus alone showed a gradual increase in H_2O_2 accumulation, with the greatest accumulation at 90 DAT. *P. indica* colonization could repress the hydrogen peroxide accumulation in leaf tissue in pre- as well as post-PYMoV inoculated plants, compared to un-colonized plants challenged by the virus, in all the intervals studied. When compared to the un-colonized plants, H_2O_2 buildup in the colonized plants challenged by the virus was minimal at 90 DAT. (Plate 17, 19, 21, and 23).

Thus, *P. indica*-colonized plants showed an increase in the chlorophyll content, total proteins and antioxidant enzymes like catalase, peroxidase, glutathione reducatse, phosphatase and SOD; and a decrease in the ROS (superoxides and hydrogen peroxide) content, when compared to the un- colonized plants, upon pre- as well as post-inoculation of PYMoV.

Table 15: Total chlorophyll content in leaves of *P. indica*-colonized black pepper plants (var. Panniyur 1), upon pre- and post-inoculation of PYMoV

Treatments	Total chlorophyll content (mg g^{-1} fresh weight)			
	15 DAT	**45 DAT**	**60 DAT**	**90 DAT**
T1 (*P. indica* alone)	1.483 ± 0.087^{a}	1.592 ± 0.515^{a}	1.743 ± 0.080^{a}	1.898 ± 0.324^{a}
T2 (PYMoV alone)	0.685 ± 0.045^{b}	0.590 ± 0.080^{b}	0.485 ± 0.097^{d}	0.415 ± 0.138^{c}
T3 (PYMoV + *P. indica*)	0.695 ± 0.121^{b}	0.675 ± 0.343^{b}	0.780 ± 0.054^{c}	0.914 ± 0.144^{b}
T8 (*P. indica* + PYMoV)	0.903 ± 0.107^{ab}	0.817 ± 0.293^{b}	1.156 ± 0.035^{b}	1.232 ± 0.050^{b}
T9 (Absolute control)	1.070 ± 0.394^{a}	1.136 ± 0.119^{ab}	1.306 ± 0.226^{b}	1.598 ± 0.109^{a}
SE (±)	0.070	0.121	0.027	0.051
CD (0.05)	0.362	0.571	0.211	0.324

Values are the means of five replications ± standard deviation; SE: Standard error; CD: Critical difference;

Superscripts with same alphabets indicate on par values and those in different alphabets indicate significant difference at 5 per cent level of significance.

Table 16: Total protein content in leaves of *P. indica*- colonized black pepper plants (var. Panniyur 1), upon pre- and post-inoculation of PYMoV

Treatments	Total protein content (mg g^{-1} fresh weight)			
	15 DAT	**45 DAT**	**60 DAT**	**90 DAT**
T1 (*P. indica* alone)	1.737 ± 0.239 [a]	1.677 ± 0.138 [b]	2.191 ± 0.419 [b]	3.061 ± 0.086 [b]
T2 (PYMoV alone)	0.910 ± 0.082 [b]	1.248 ± 0.186 [c]	1.169 ± 0.168 [d]	1.532 ± 0.211 [cd]
T3 (PYMoV + *P. indica*)	1.176 ± 0.151 [b]	1.015 ± 0.970 [c]	1.716 ± 0.196 [c]	2.047 ± 0.332 [c]
T8 (*P. indica* + PYMoV)	1.603 ± 0.186 [a]	2.627 ± 0.162 [a]	4.133 ± 0.195 [a]	5.360 ± 0.587 [a]
T9 (Absolute control)	0.874 ± 0.207 [b]	0.706 ± 0.070 [d]	0.802 ± 0.123 [d]	0.996 ± 0.384 [d]
SE (±)	0.077	0.060	0.111	0.169
CD (0.05)	0.330	0.258	0.442	0.658

Values are the means of five replications ± standard deviation; SE: Standard error; CD: Critical difference;

Superscripts with same alphabets indicate on par values and those in different alphabets indicate significant difference at 5 per cent level of significance.

Table 17: Catalase activity in leaves of *P. indica*- colonized black pepper plants (var. Panniyur 1), upon pre- and post-inoculation of PYMoV

Treatments	Total catalase activity (enzyme units min^{-1} g^{-1}fw)			
	15 DAT	**45 DAT**	**60 DAT**	**90 DAT**
T1 (*P. indica* alone)	464.148 ± 10.516^{b}	504.175 ± 14.889^{ab}	513.514 ± 10.811^{b}	525.817 ± 6.453^{b}
T2 (PYMoV alone)	371.134 ± 18.793^{c}	400.092 ± 5.331^{c}	394.919 ± 7.733^{d}	403.184 ± 6.617^{d}
T3 (PYMoV + *P. indica*)	495.608 ± 8.240^{c}	481.127 ± 8.178^{c}	491.529 ± 9.829^{b}	455.524 ± 6.988^{b}
T8 (*P. indica* + PYMoV)	558.047 ± 5.708^{a}	555.374 ± 22.774^{a}	524.422 ± 7.861^{a}	494.496 ± 12.192^{a}
T9 (Absolute control)	94.351 ± 10.665^{d}	108.912 ± 17.873^{d}	117.823 ± 12.883^{e}	103.573 ± 13.720^{e}
SE (±)	5.250	5.023	4.428	3.644
CD (0.05)	22.649	20.418	24.786	15.739

Values are the means of five replications ± standard deviation; SE: Standard error; CD: Critical difference;

Superscripts with same alphabets indicate on par values and those in different alphabets indicate significant difference at 5 per cent level of significance.

Table 18: Peroxidase activity in leaves of *P. indica*- colonized black pepper plants (var. Panniyur 1), upon pre- and post-inoculation of PYMoV

Treatments	Total peroxidase activity (µg g^{-1}fw)			
	15 DAT	**45 DAT**	**60 DAT**	**90 DAT**
T1 (*P. indica* alone)	9.953 ± 0.114^{a}	9.926 ± 0.918^{a}	10.232 ± 0.441^{a}	10.465 ± 0.483^{b}
T2 (PYMoV alone)	8.251 ± 0.262^{c}	7.996 ± 0.036^{c}	8.556 ± 0.418^{c}	7.991 ± 0.115^{d}
T3 (PYMoV + *P. indica*)	8.683 ± 0.129^{b}	9.170 ± 0.453^{b}	9.365 ± 0.409^{b}	9.182 ± 0.319^{c}
T8 (*P. indica* + PYMoV)	10.208 ± 0.177^{a}	10.420 ± 0.092^{a}	10.700 ± 0.197^{a}	11.338 ± 0.243^{a}
T9 (Absolute control)	5.023 ± 0.086^{d}	4.672 ± 0.389^{d}	5.044 ± 0.075^{d}	5.963 ± 0.059^{e}
SE (±)	0.072	0.112	0.156	0.124
CD (0.05)	0.301	0.546	0.681	0.124

Values are the means of five replications ± standard deviation; SE: Standard error; CD: Critical difference;

Superscripts with same alphabets indicate on par values and those in different alphabets indicate significant difference at 5 per cent level of significance.

Table 19: Glutathione reductase activity in leaves of *P. indica*-colonized black pepper plants (var. Panniyur 1), upon pre- and post-inoculation of PYMoV

Treatments	Total glutathione reductase activity (enzyme units min^{-1} g^{-1}fw)			
	15 DAT	**45 DAT**	**60 DAT**	**90 DAT**
T1 (*P. indica* alone)	205.737 ± 10.133 [a]	218.484 ± 9.490 [a]	227.831 ± 8.090 [b]	213.870 ± 22.067 [a]
T2 (PYMoV alone)	105.013 ± 11.251 [c]	123.133± 6.451 [c]	107.974 ± 8.790 [d]	113.072 ± 13.071 [b]
T3 (PYMoV + *P. indica*)	137.579± 17.709 [b]	167.386 ± 7.079 [b]	186.667 ± 9.892 [c]	193.922 ± 7.354 [a]
T8 (*P. indica* + PYMoV)	196.026± 8.637 [a]	229..347 ± 27.966 [a]	249.701 ± 14.651 [a]	216.340 ± 11.471 [a]
T9 (Absolute control)	50.146± 11.206 [d]	58.173 ± 3.511 [d]	66.167 ± 8.104 [e]	61.026 ± 6.341 [c]
SE (±)	5.271	4.874	9.906	5.394
CD (0.05)	22.177	25.419	18.568	24.184

Values are the means of five replications ± standard deviation; SE: Standard error; CD: Critical difference;

Superscripts with same alphabets indicate on par values and those in different alphabets indicate significant difference at 5 per cent level of significance.

Table 20: Glutamate synthase activity in leaves of *P. indica*- colonized black pepper plants (var. Panniyur 1), upon pre- and post-inoculation of PYMoV

Treatments	Total glutamate synthase activity (enzyme units min^{-1} g^{-1}fw)			
	15 DAT	**45 DAT**	**60 DAT**	**90 DAT**
T1 (*P. indica* alone)	10.085 ± 1.030 [a]	10.730 ± 2.025 [a]	6.500 ± 0.201 [a]	10.624 ± 1.196 [a]
T2 (PYMoV alone)	8.343 ± 0.221 [b]	8.234 ± 0.266 [b]	8.056 ± 0.081 [b]	8.041 ± 0.101 [b]
T3 (PYMoV + *P. indica*)	8.382 ± 0.143 [b]	8.295 ± 0.081 [bc]	7.923 ± 0.148 [b]	8.021 ± 0.102 [b]
T8 (*P. indica* + PYMoV)	8.624 ± 0.033 [b]	8.635± 0.033 [b]	8.567 ± 0.099 [b]	8.561 ± 0.070 [b]
T9 (Absolute control)	2.821 ± 0.043 [c]	3.286 ± 0.323 [c]	3.717 ± 0.170 [c]	3.626 ± 0.085 [c]
SE (±)	0.131	0.071	0.261	0.164
CD (0.05)	0.866	1.684	1.647	0.983

Values are the means of five replications ± standard deviation; SE: Standard error; CD: Critical difference;

Superscripts with same alphabets indicate on par values and those in different alphabets indicate significant difference at 5 per cent level of significance.

Table 21: Phosphatase activity in leaves of *P. indica*-colonized black pepper plants (var. Panniyur 1), upon pre- and post-inoculation of PYMoV

Treatments	Total phospatase activity (milli moles of pNP released min^{-1} g^{-1}fw)			
	15 DAT	**45 DAT**	**60 DAT**	**90 DAT**
T1 (*P. indica* alone)	39.210 ± 4.229^{c}	36.801 ± 5.430^{c}	46.435 ± 5.690^{c}	40.415 ± 3.030^{d}
T2 (PYMoV alone)	53.259 ± 5.518^{b}	53.661 ± 6.179^{b}	59.682 ± 3.030^{b}	54.123 ± 5.438^{c}
T3 (PYMoV + *P. indica*)	60.083 ± 8.458^{ab}	59.682 ± 4.228^{ab}	67.308 ± 3.679^{a}	64.097 ± 3.612^{b}
T8 (*P. indica* + PYMoV)	68.104 ± 4.240^{a}	65.703 ± 5.430^{a}	73.329 ± 1.839^{a}	72.125 ± 3.678^{a}
T9 (Absolute control)	30.361 ± 5.519^{c}	36.400 ± 2.085^{c}	37.605 ± 2.408^{d}	38.006 ± 3.871^{d}
SE (±)	2.501	2.089	1.489	1.756
CD (0.05)	10.555	8.890	6.523	7.291

Values are the means of five replications ± standard deviation; SE: Standard error; CD: Critical difference;

Superscripts with same alphabets indicate on par values and those in different alphabets indicate significant difference at 5 per cent level of significance.

Table 22: Superoxide dismutase activity in leaves of *P. indica*- colonized black pepper plants (var. Panniyur 1), upon pre- and post-inoculation of PYMoV

Treatments	Total SOD activity (enzyme units min^{-1} g^{-1}fw)			
	15 DAT	**45 DAT**	**60 DAT**	**90 DAT**
T1 (*P. indica* alone)	343.661 ± 11.244 [a]	414.735 ± 49.780 [a]	440.119 ± 6.765 [b]	450.110 ± 5.867 [b]
T2 (PYMoV alone)	140.760 ± 5.742 [d]	331.690 ± 34.832 [d]	301.816 ± 6.795 [d]	291.574 ± 16.407 [d]
T3 (PYMoV + *P. indica*)	245.553 ± 1.641 [c]	338.050 ± 19.557 [c]	336.299 ± 5.708 [c]	342.941 ± 5.239 [c]
T8 (*P. indica* + PYMoV)	275.547 ± 9.926 [b]	493.101 ± 2.740 [b]	498.702 ± 2.799 [a]	551.846 ± 5.689 [a]
T9 (Absolute control)	75.406± 6.653 [c]	147.784 ± 21.213 [c]	100.853 ± 4.496 [e]	123.429 ± 3.387
SE (±)	3.141	11.453	2.378	3.273
CD (0.05)	20.346	54.747	10.063	15.752

Values are the means of five replications ± standard deviation; SE: Standard error; CD: Critical difference;

Superscripts with same alphabets indicate on par values and those in different alphabets indicate significant difference at 5 per cent level of significance.

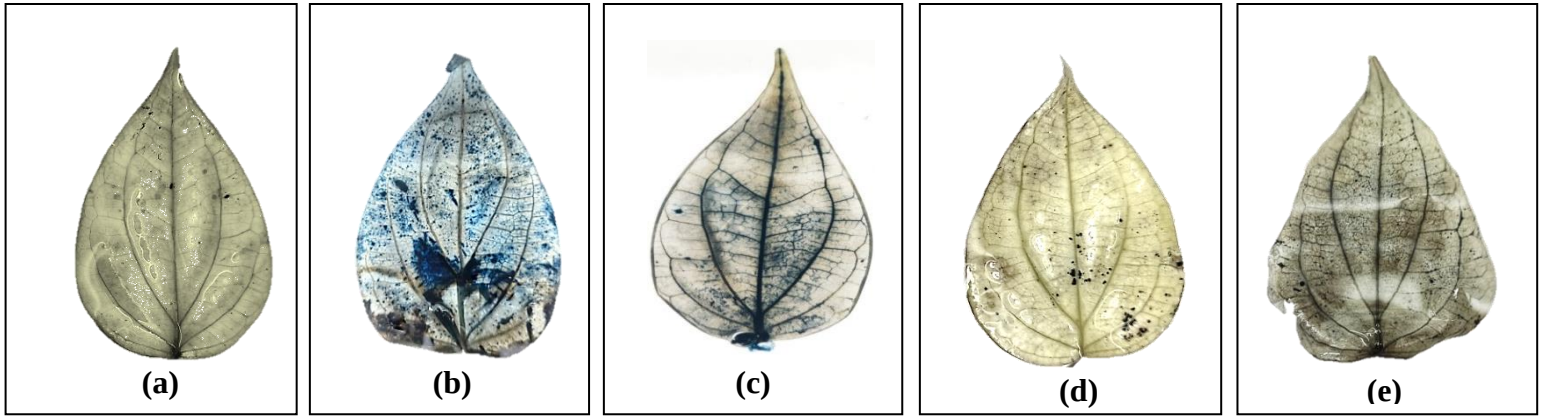


Plate 16 : Detection of superoxides by NBT staining of black pepper leaves at 15 days after treatments: (a) *P. indica* alone (b) PYMoV alone (c) PYMoV + *P. indica* (d) *P. indica* + PYMoV (e) Absolute control

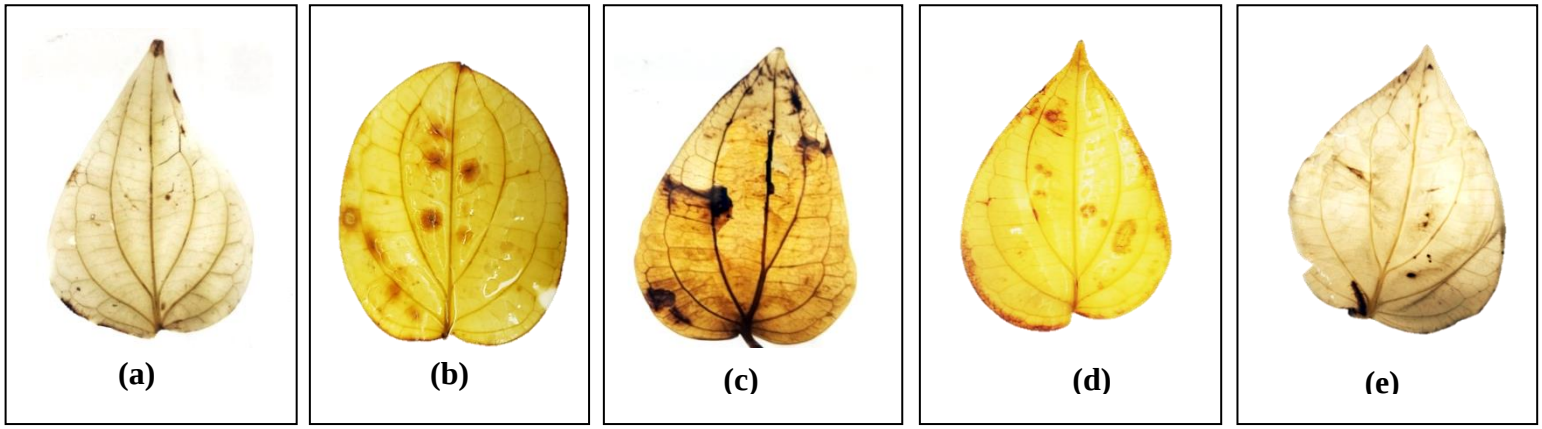


Plate 17: Detection of hydrogen peroxide by DAB staining of black pepper leaves, 15 days after treatments: (a) *P. indica* alone (b) PYMoV alone (c) PYMoV + *P. indica* (d) *P. indica* + PYMoV (e) Absolute control

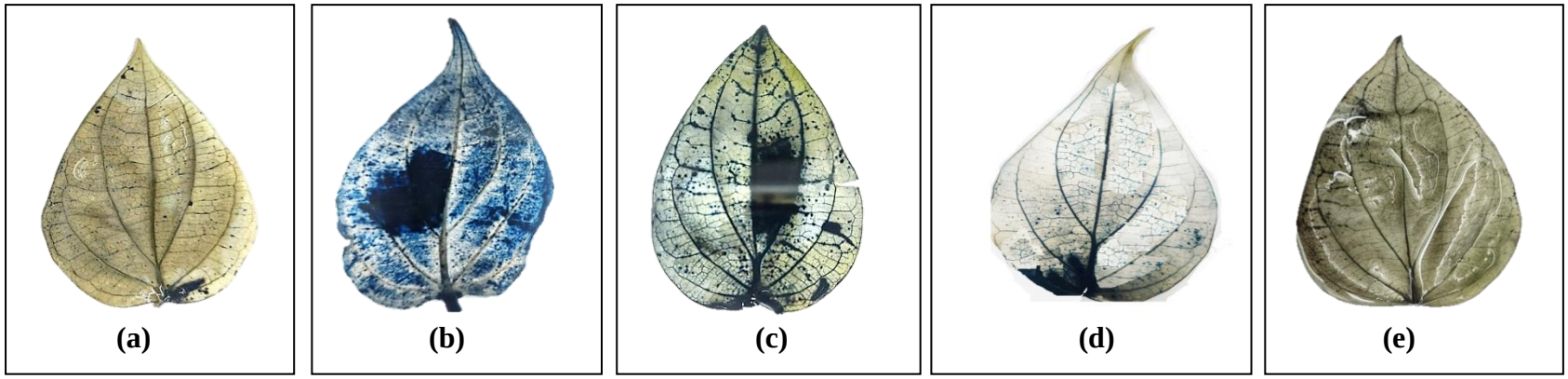


Plate 18: Detection of superoxides (O_2^-) by NBT staining of black pepper leaves, 45 days after treatments: (a) *P. indica* alone (b) PYMoV alone (c) PYMoV + *P. indica* (d) *P. indica* + PYMoV (e) Absolute control

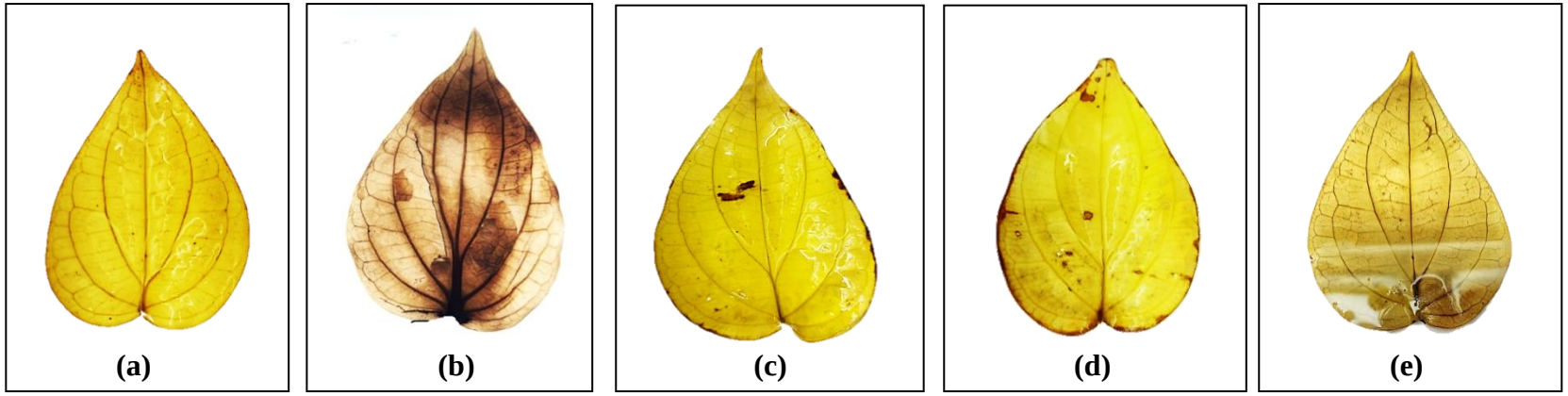


Plate 19: Detection of hydrogen peroxide by DAB staining of black pepper leaves, 45 days after treatments: (a) *P. indica* alone (b) PYMoV alone (c) PYMoV + *P. indica* (d) *P. indica* + PYMoV (e) Absolute control

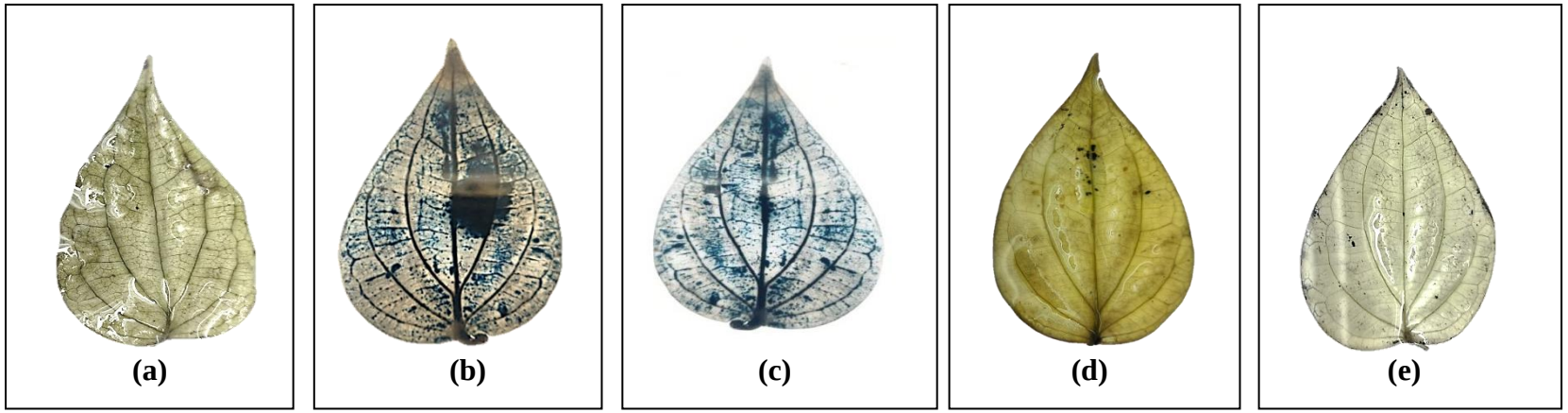


Plate 20: Detection of superoxides (O_2^-) by NBT staining of black pepper leaves, 60 days after treatments: (a) *P. indica* alone (b) PYMoV alone (c) PYMoV + *P. indica* (d) *P. indica* + PYMoV (e) Absolute control

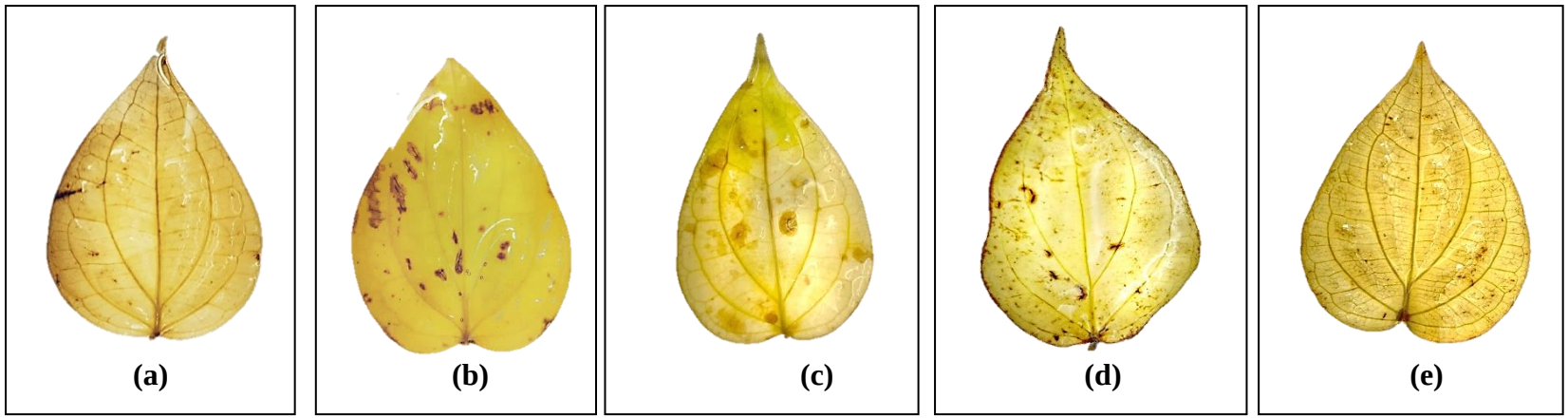


Plate 21: Detection of hydrogen peroxide by DAB staining of black pepper leaves, 60 days after treatments: (a) *P. indica* alone (b) PYMoV alone (c) PYMoV + *P. indica* (d) *P. indica* + PYMoV (e) Absolute control

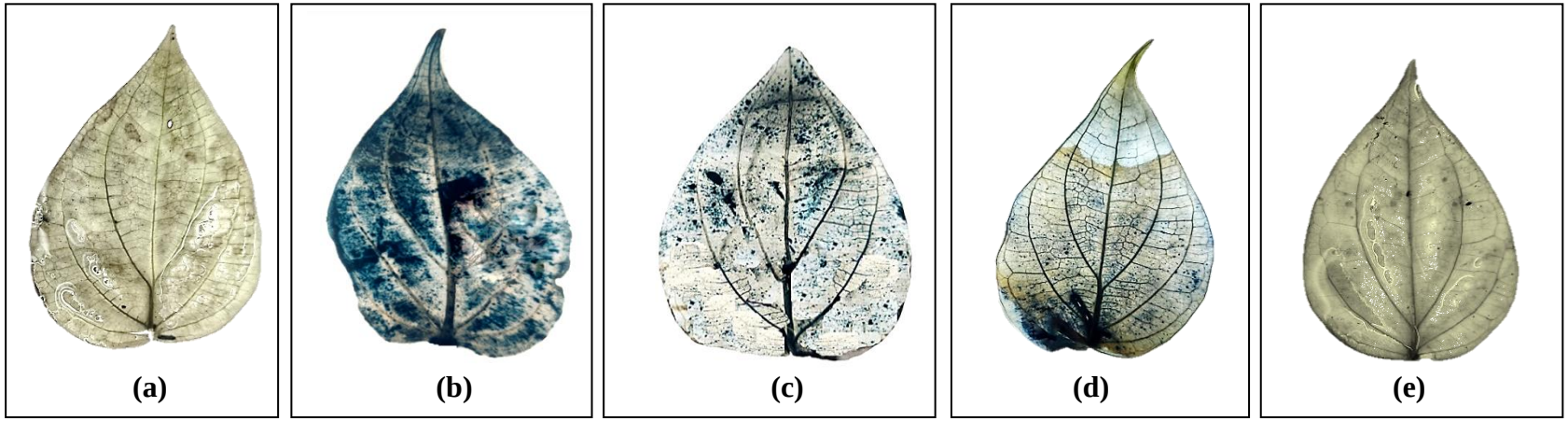


Plate 22: Detection of superoxides (O_2^-) by NBT staining of black pepper leaves, 90 days after treatments: (a) *P. indica* alone (b) PYMoV alone (c) PYMoV + *P. indica* (d) *P. indica* + PYMoV (e) Absolute control

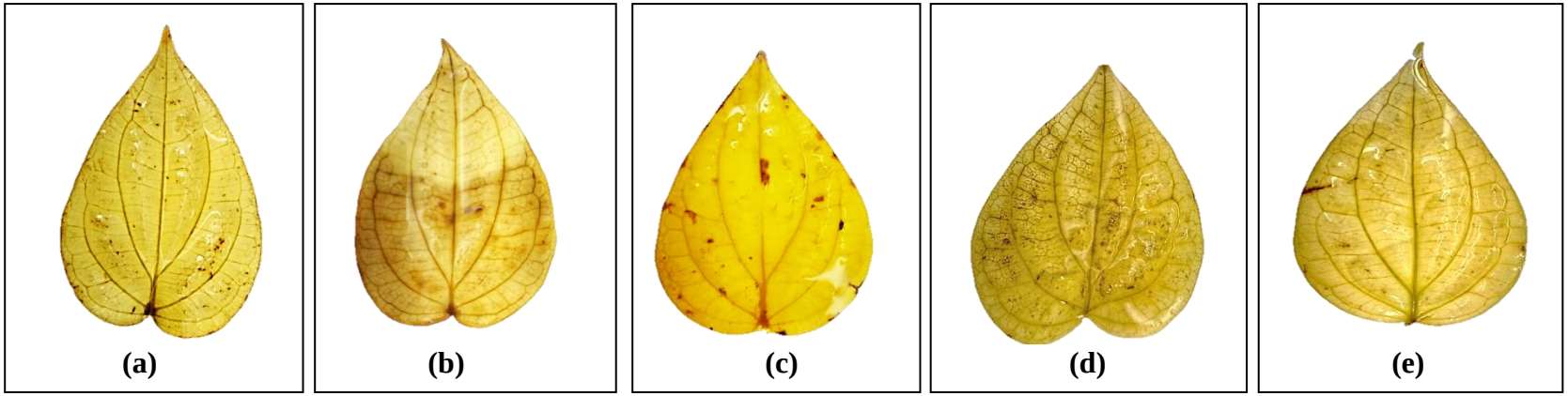


Plate 23: Detection of hydrogen peroxide by DAB staining of black pepper leaves, 90 days after treatments: (a) *P. indica* alone (b) PYMoV alone (c) PYMoV + *P. indica* (d) *P. indica* + PYMoV (e) Absolute control

Discussion

5. DISCUSSION

5.1. SUSCEPTIBILITY OF BLACK PEPPER PLANTS TO PYMoV

A survey was conducted in the Instructional Farm, College of Agriculture, Vellayani to identify and collect PYMoV-infected black pepper cuttings. All the surveyed black pepper varieties were susceptible to PYMoV infection, with disease incidence ranging from 10 to 90 per cent, endorsing that even the superior hybrids are not resistant to PYMoV infection. Upon categorizing the surveyed varieties based on their reaction to PYMoV infection, var. Karimunda was highly susceptible, Panniyur 6 was moderately susceptible and Panniyur 1, Panniyur 2, Panniyur 3, Panniyur 4, Panniyur 5 and Panniyur 7 were moderately resistant.

Such a high incidence of the disease even in nursery conditions is mainly due to the use of virus-infected planting materials for vegetative propagation. Farmers and stakeholders are either careless or ignorant about the symptoms of the virus infection while selecting the mother plants for propagation. Also, observing the runner shoots alone for the symptoms of the virus may not suffice, as the vines with apparently healthy runner shoots may have symptoms of the virus in the orthotropic or plagiotropic shoots, which also have a systemic spread inside the plant. When such runner shoots are used for propagation, the resulting progeny plants will be harboring the virus as well. Therefore, comprehensive examination of the whole plant is a must before selecting the mother vines for propagation, as black pepper is a perennial crop. Once the crop is infected, it is very difficult or even impossible to eliminate the virus from the vines. Use of disease-free mother plants for vegetative propagation itself can control the disease to a great extent, as it is the primary source of disease spread. Secondary spread through insect vectors (*Ferrisia virgata* and *Planococcus citri*) is not of much significance, due to the slow movement of these vectors in the field.

Visual observation for symptoms is a good criterion for selecting disease-free mother plants. But depending on the growth and environmental conditions, masking of symptoms may occur, and the virus-infected plants may appear apparently healthy without showing any

visible symptoms. Under favorable conditions like high temperature and nutrient deprivation, the symptoms get expressed, leading to yield loss. Hence, mother plants used for vegetative propagation should be periodically indexed for viruses and the cuttings should be raised under insect-proof conditions. Indexing may be done by grafting parts of the suspected vines onto black pepper varieties that are highly susceptible to PYMoV infection, such as var. Karimunda. If no symptoms are produced on the indicator plant, the vine under inspection may be approved as virus- free. This technique, which is being effectively utilized in the indexing of citrus plants for *Citrus tristeza virus* using Kazgi lime (*Citrus aurantifolia*) as the indicator plant (Roistacher, 1991), may be employed here also.

Serological techniques like ELISA are also not trustworthy, as they show false-negative results, owing to the low titre of the virus inside the plants. This necessitates the use of sensitive techniques like PCR for fool-proof detection (Bhat *et al*., 2009). Virus-free mother vines may be propagated vegetatively under insect-proof conditions to ensure good health of the progeny.

To date, genetic resistance and control of vectors are the most widespread strategies against viral diseases. But, so far no black pepper variety has been reported to be resistant to PYMoV infection. This might be due to the lack of virus-resistance genes in black pepper. Also vector control too may not be enough for the management of PYMoV, as vectors have negligible role in the spread of the disease. Since the commercial high yielding black pepper varieties lack the ability to resist the virus infection naturally, exogenous agents such as beneficial endophytes if administered properly, can be real life-savers.

5.2. *P. indica*-COLONIZATION IN GROWTH PROMOTION OF BLACK PEPPER PLANTS

Beneficial effects of *P. indica* on various growth parameters of a wide range of crops have been reported. But the idea about what exactly are the reasons behind these effects is still ambiguous though few reasons are well stated.

In the current study also, significant increase in shoot and root biomass could be observed in *P. indica*-colonized black pepper plants compared to the control plants. The *P. indica*-colonized plants had longer shoots, more leaves, greater leaf area, and more roots than the control plants. Increase in root biomass due to enhanced production of lateral roots observed in the colonized plants increases the surface area for absorption of nutrients. Also, increase in the number of leaves and leaf area boosts the photosynthetic efficiency, thus promoting growth. During the study, a significant increase in total chlorophyll content was observed in the *P. indica*-colonized black pepper plants compared to the control plants (Fig. 3). Higher chlorophyll content might also have enhanced the photosynthesis rate and in turn the carbohydrate synthesis, which also might have accelerated the growth rate of the colonized plants.

Assimilation of nutrients like nitrogen and phosphorus in exchange of carbon from photosynthates has been proposed as a reason for this endophyte-mediated growth promotion. *P. indica* can extract, mobilize, and transport nitrogen (N), phosphorus (P), potassium (K), sulphur (S), and magnesium (Mg), as well as minor nutrients such as iron (Fe), zinc (Zn), manganese (Mn), and copper (Cu). Malla *et al.* (2004) reported that *P. indica* contains high amount of acid phosphatase enzyme, which helps in solubilizing the phosphate in soil and makes it available to the plant. Further, Shahollari *et al.* (2007) demonstrated that growth promotion in *P. indica*-colonized Arabidopsis plants could be attributed to the increased uptake of phosphorus from the growth medium. Yadav *et al*. (2010) identified *P. indica*-phosphate transporter (PiPT) gene, which helps to transport phosphates from the soil to the plant as a result of *P. indica* colonization and this PiPT protein was later purified and characterized by Pedersen *et al*. in 2013. Sherameti *et al.* (2005) concluded that *P. indica*-mediated growth promotion in tobacco and Arabidopsis was due to the up-regulation of a gene involved in uptake of nitrate (*Nia2*).

The role of phytohormones in *P. indica*-mediated growth promotion has also been investigated. There are reports suggesting that auxins, gibberellins and cytokinins play crucial roles in *P. indica*-mediated growth promotion in Arabidopsis, barley and Chinese

cabbage (Sirrenberg *et al.* 2007; Vadassery *et al.* 2008; Lee *et al.* 2011). Auxins are involved in cell expansion, cell division, and cell differentiation and play a key role in root growth, tropism, apical dominance, and plant senescence (Davies, 2004). They have a significant impact on root morphology by reducing root elongation, boosting the development of lateral roots, and enhancing adventitious root formation. Sirrenberg *et al.* (2007) reported that *P. indica* produced IAA in liquid culture and this fungal auxin is responsible for promoting root growth in plants. Vadassery *et al*. (2008) reported that cytokinins, particularly trans-zeatin, play a critical role in *P. indica*-mediated growth promotion in Arabidopsis. Different cytokinin receptor genes *viz.* CRE1, AHK2, AHK3, the cytokinin-responsive gene ARR5, and transzeatin cytokinin biosynthetic genes were considerably elevated in the colonized roots, indicating that cytokinin has a favourable role in *P. indica*-mediated Arabidopsis growth promotion. *P. indica* inhibits ethylene synthesis in plants, which also helps in promoting plant growth (Barazani *et al*., 2007).

5.3. *P. indica*-COLONIZATION IMPEDES PYMoV INFECTION IN BLACK PEPPER PLANTS

There are several reports suggesting that *P. indica* can act as a bio-protector too, against various abiotic and biotic stresses (Waller *et al*., 2005; Serfling *et al*., 2007; Kumar *et al*., 2009; Fakhro *et al*., 2010; Johnson *et al*., 2013). Results of the present study also confirm that treatment with *P. indica* is effective in significantly reducing the intensity and severity of PYMoD in black pepper. Black pepper plants treated prophylactically as well as curatively with *P. indica* were able to recover to a certain extent, the loss in biomass caused by PYMoV infection and symptoms expressed compared to the non-colonized plants upon challenge inoculation of the virus (Fig. 1 and 2). PCR amplification further demonstrated that viral titer was lowest in *P. indica*-colonized plants that were post-inoculated with the virus, followed by pre-inoculated plants that were subsequently colonized with *P. indica*, and highest in the un-colonized plants that were inoculated with the virus alone (Plate 15). This throws light into the fact that *P. indica*-colonization is somehow hindering the replication of PYMoV inside the plants and the exact mechanism by which this is brought about is beyond

the scope of this study. However, reduced titer value of the virus can be correlated to the reduced symptom expression in the colonized plants upon artificial inoculation of the virus.

Further, among the post-inoculation treatments, co-cultivation with *P. indica* followed by artificial inoculation of the virus after 30 days offered a better control of PYMoV infection, compared to artificial inoculation of the virus at zero, two, seven and 15 days after co-cultivation. Thus it can be inferred that, co-cultivation of rooted cuttings of black pepper (obtained from disease-free mother plants) with *P. indica* and keeping them under insect-proof conditions for around 30 days (for successful establishment of the fungus inside the root cortex) and then planting them in the main field is a reliable and chemical-free method to control PYMoD in black pepper.

The results from the study revealed that PYMoV infection resulted in a four-fold reduction in total chlorophyll content in the non-colonized plants compared to the absolute control plants. The reduction in chlorophyll content in virus-infected plants may be due to the stimulation of specific cellular enzymes such as chlorophyllase or due to the effect of the virus on pigment synthesis (Balachandran *et al*., 1997). Whereas, in the case of *P. indica*-colonized plants that were pre- and post-inoculated with the virus, the reduction in chlorophyll content was only 1.7 and 1.3 times compared to the absolute control plants, at 90 DAT (Fig. 3); indicating that *P. indica*-colonization could mitigate the above mentioned harmful effects of PYMoV infection on chlorophyll. Furthermore, *P. indica*-colonized plants that were post- inoculated with the virus showed 31 per cent more shoot length, 60 per cent more number of leaves and 65 per cent more leaf area than the non-colonized diseased plants, at 90 days after treatments. Similarly, plants pre-inoculated with the virus and then colonized by *P. indica* showed 24 per cent more shoot length, 50 per cent more number of leaves and 45 per cent more leaf area than the non-colonized control plants, on graft inoculation of PYMoV. Higher chlorophyll content, along with increased leaf area and number of leaves in *P. indica*-colonized plants might have resulted in increased photosynthetic efficiency in the colonized plants even after being challenged by the virus and thus reduced the loss in biomass and symptoms caused due to the infection.

The exact mechanism by which *P. indica*-colonization leads to the viral disease suppression is not yet clearly understood, but several other possible reasons have also been investigated. There are reports suggesting that *P. indica* fights foliar infections by developing systemic resistance in plants, which is similar to the well-known methods for plant growth-promoting rhizobacteria (van Wees *et al.,* 2008). *P. indica* once colonized in plants, provides systemic bioprotection for the duration of the crop. When *P. indica* interacts with plant roots, potential signaling pathways are activated, resulting in alterations in the plant transcriptome, proteome and metabolome, as well as phytohormones that have systemic effects throughout the plant (Johnson *et al*., 2014). Beneficial fungal and bacterial colonization in plants activates the innate immune system transiently in order to counteract different pathogens via a multi-layered innate immune system that relies on Ca^{2+} and ROS signalling, activation of Ca^{2+}-dependent protein and MAP kinases (CDPK, MAPK) and rapid production of stress hormones like jasmonic acid, salicylic acid, ethylene and abscissic acid (Boller and Felix, 2009).

5.4. ROLE OF ROS AND ANTIOXIDANT ENZYMES IN *P. indica*-MEDIATED RESISTANCE TO PYMoV

Plants have evolved a number of strategies for avoiding pathogenic infection. The rapid generation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide anions (O_2^-) is the first line of defense against these obligate intracellular parasites (Bolwell and Wojtaszek, 1997). In the current study also, the results of NBT and DAB staining (Plate 16 to 23) revealed that ROS like superoxides and hydrogen peroxide accumulation were the highest in black pepper plants inoculated with the virus alone and this higher levels of ROS over a long period of time might be the reason for the highest disease severity and weakened growth of the plants infected with the virus alone (Plate 14). Similar results were obtained when Riedle-Bauer (2000) investigated oxidative stress in *Cucumis sativus* infected with *Cucumber mosaic virus* and in *Cucurbita pepo* plants infected with *Zucchini yellow mosaic virus*, concluding that virus-enhanced peroxidation via the formation

of ROS is involved in the development of mosaic and yellowing symptoms in virus-infected tissues.

Moreover, in the absence of PYMoV infection, no increase in hydrogen peroxide as well as superoxide anion concentration was observed in the leaves of absolute control plants as well as healthy plants colonized by *P. indica.* This indicates that PYMoV infection was solely responsible for the accumulation of hydrogen peroxide and superoxide anion in black pepper leaves, and *P. indica* did not cause any ROS accumulation in the leaves.

In infected plants, ROS have the potential to act as long-range signaling molecules that alert the plants, orchestrate programmed cell death (PCD), and confinement of invading pathogens to the infected parts. However, if their concentration exceeds a certain level, cellular homeostasis may be disrupted. Increased ROS generation can damage cells by causing lipid peroxidation, protein oxidation, nucleic acid degradation, enzyme inhibition, and incite PCD pathways, all of which can lead to cell death (Dreyer and Dietz, 2018). Plants must maintain a perfect balance between ROS formation and ROS-scavenging pathways in order to effectively use ROS as a signaling molecule and as a result, minimize the possible harmful consequences of ROS.

Among the several mechanisms by which *P. indica* brings about disease suppression in plants, boosting the antioxidant defense system of plants and in turn counteracting the detrimental effects of ROS produced by the plant-pathogen interaction is thought to be a crucial one (Waller *et al.,* 2005; Serfling *et al.*, 2007; Kumar *et al.,* 2009). Hence in the current study, to check whether *P. indica*-colonization plays any role in modulating the biochemical responses of black pepper plants to PYMoV infection, the activity of various enzymes such as catalase, peroxidase, glutathione reductase, glutamate synthase, phosphatase, and superoxide dismutase, which are known to play important roles in plant antioxidant defense systems, were measured at 15, 45, 60, and 90 days after treatments. It was observed that PYMoV infection significantly increased the activities of antioxidant enzymes in black pepper plants, compared to the healthy plants. Similar results were obtained by Sofy *et al* (2020), where they could observe increased the activities of

antioxidant enzymes (SOD, POX, PPO, LOX, APX, GR, chitinase and β-1, 3 glucanase) in CMV infected cucumber plants compared to those in healthy plants. But prophylactic as well as curative treatment with *P. indica* resulted in even more enhanced activity of these enzymes in the infected plants compared to the plants infected with the virus alone.

SODs are the most powerful intracellular enzymatic antioxidants, and the only plant eznymes capable of scavenging O_2^-. It is a metalloenzyme whose early activation is critical in the first line of plant defense, as it catalyzes the dismutation of superoxide radicals into less toxic H_2O_2 and O_2, thus preventing oxidative damage. The enzyme is localized in the cytosol, chloroplast, mitochondria and peroxisomes (Mittler *et al*., 2004). PYMoV infection resulted in significant increase in SOD activity in the leaf tissues of black pepper plants over the healthy plants (Fig. 10). Following pathogen infection, an increase in SOD activity may be necessary to catalyze the production of less reactive H_2O_2 and avoid the buildup of more reactive O_2^- radicals. SOD activity may help to lower the chance of an OH^- radical forming as a result of a Fenton-type reaction, which could lead to increased superoxide production (Halliwell and Gutteridge, 1989). In the *P. indica*-colonized black pepper plants pre- as well as post-inoculation of PYMoV, SOD activity was around two-fold higher than that in the plants inoculated with the virus alone (Table 22). This increase in SOD activity in the colonized plants is reflected in the reduced accumulation of superoxide radicals, as evident from the results of NBT staining (Plate 15, 17, 19 and 21).

Catalases and peroxidases catalyze the dismutation of H_2O_2 into H_2O and O_2. One catalase molecule can convert around 6 million H_2O_2 molecules per minute. Catalases are localized in mitochondria, peroxisomes and glyoxisomes (Dat *et al.*, 2000); perform their activity in the presence of high concentrations of H_2O_2, without the help of any reductants and is thus an energy-efficient way to remove H_2O_2. Peroxides on the other hand, owing to their higher affinity for the oxidizing substrate and a more widespread presence in various subcellular compartments, can also act at low concentrations (van Breusegem *et al*., 2001). Peroxidases-driven reactions use low relative molecular mass antioxidants like glutathione and ascorbate as electron donors, so removing H_2O_2 via this pathway could be a very energy-

intensive reaction for the cell because it uses important molecules from the environment: two GSH molecules are consumed for each H_2O_2 molecule removed (Kohen and Nyska, 2002). This is exactly what might have happened in this study also, wherein *P. indica*-colonization triggered an initial surge of catalase activity during the early phases of infection, when H_2O_2 levels were the highest, and later resulted in enhanced peroxidase activity when H_2O_2 became less available (Fig. 3 and 4). Increased activity of catalases and peroxidases might be the reason for reduced accumulation of H_2O_2 in *P. indica*-colonized PYMoV-infected plants, as evident from the DAB staining.

GR is an oxidoreductase that plays a crucial role in antioxidant defense system by catalyzing the NADPH dependent reduction of the disulphide bond of oxidized glutathione (GSSG), thus sustaining the reduced status of glutathione (GSH), a non-enzymatic antioxidant engaged in numerous metabolic regulation and antioxidative processes in plants. GR is predominantly found in chloroplasts with small amounts occurring in the mitochondria and cytosol (Mittler *et al*., 2004). Increased levels of GR might have increased the concentration of GSH and hence reduced the oxidative stress.

The results obtained from the study thus states that PYMoV infection brings about a substantial increase in ROS accumulation and elevated levels of antioxidant enzyme activity. But in normal sense this is not expected, as increase in antioxidant activity should normally lead to decrease in ROS accumulation. A possible explanation for this observation could be that since viruses are obligate parasites they will not permit PCD through excess ROS production, as they require living cells for their survival. So they activate the defense system of the plants to produce antioxidants that scavenge the ROS produced. In response to this, plants further increase the production of ROS with the motive of eliminating the virus at any cost. This might be the reason for increase in ROS as well as antioxidant enzymes in the plants at early stages. But since ROS accumulation is harmful to the plants as well, increase in ROS beyond a threshold level automatically activates the antioxidant defense system of the plant. But in the virus-infected plants, this elevation in antioxidant activity is insufficient to cope with the greater levels of ROS produced during the infection; and thus the plants

succumb to the virus infection. *P. indica*-colonization elevates the antioxidant levels in the plants to a point where most of the ROS produced can be efficiently scavenged. Also there are several lines of evidence stating that *P. indica* represses the expression of several ROS marker genes such as *RRTF1* (Redox Responsive Transcription Factor 1) and *OXI1* (Oxidative Signal-Inducible 1), thus inhibiting the generation of ROS in plants (Camehl *et al*., 2011). Thus *P. indica* helps the plants to mitigate the deleterious effects of ROS by inhibiting their generation.

Thus the study reveals that, co-cultivation of rooted cuttings of black pepper (obtained from disease-free mother plants) with *P. indica* and keeping them under insect-proof conditions for around 30 days (for successful establishment of the fungus inside the root cortex) and then planting them in the main field is a reliable and chemical-free method to control PYMoD in black pepper.

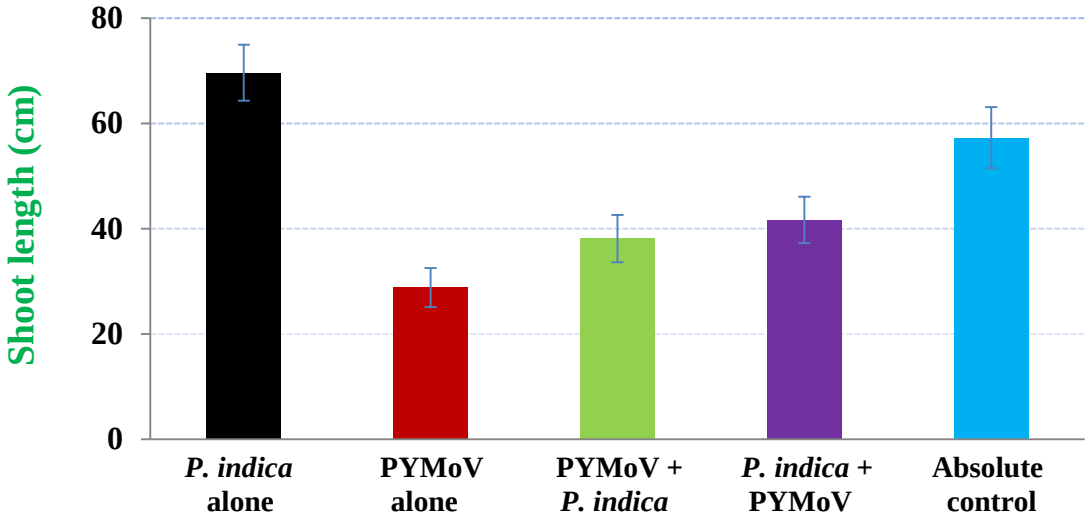


Figure 1: Effect of *P. indica*-colonization on shoot length of black pepper plants (var. Panniyur 1), upon pre- and post-inoculation of PYMoV, at 90 days after treatments

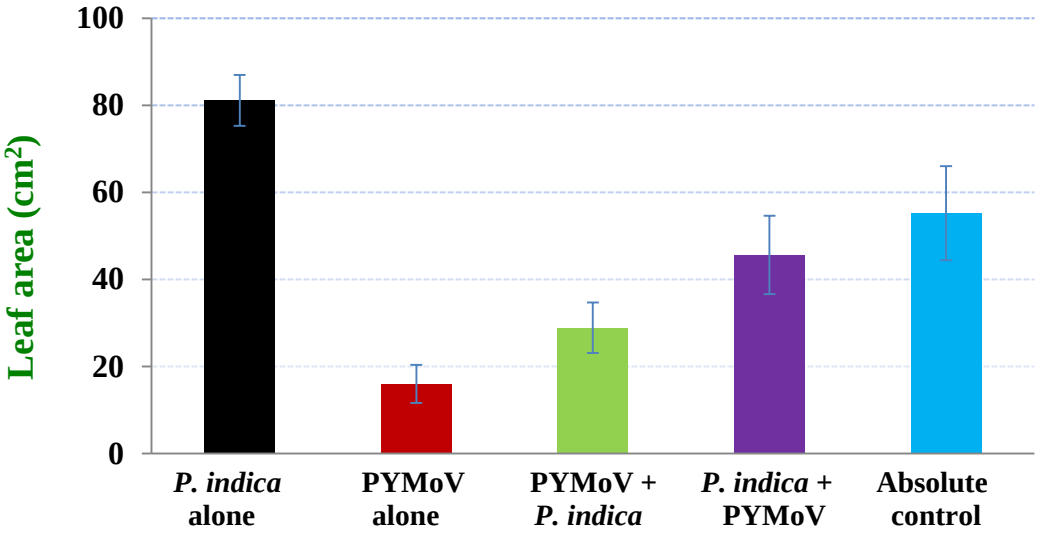


Figure 2: Effect of *P. indica*-colonization on leaf area of black pepper plants (var. Panniyur 1), upon pre- and post-inoculation of PYMoV, at 90 days after treatments

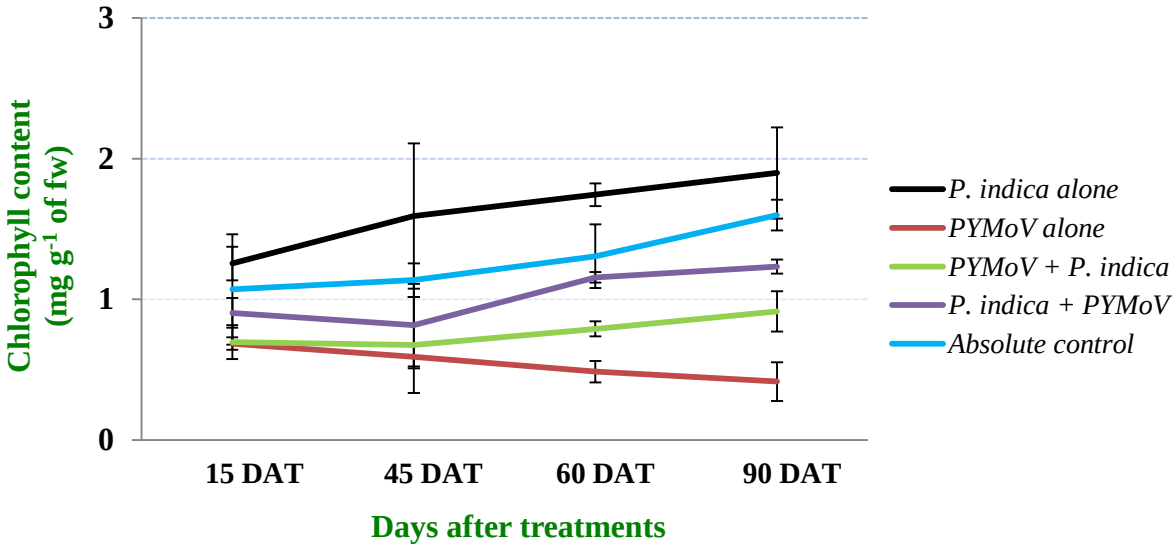


Figure 3: Total chlorophyll content in leaves of *P. indica*- colonized black pepper plants (var. Panniyur 1), upon pre- and post- inoculation of PYMoV

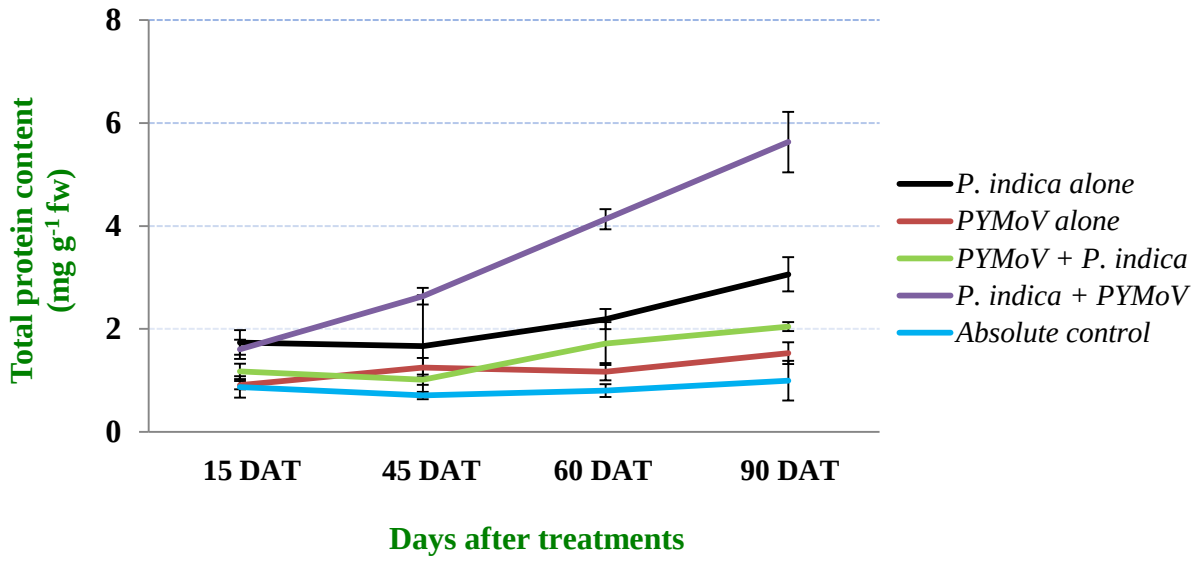


Figure 4: Total protein content in leaves of *P. indica*- colonized black pepper plants (var. Panniyur 1), upon pre- and post- inoculation of PYMoV

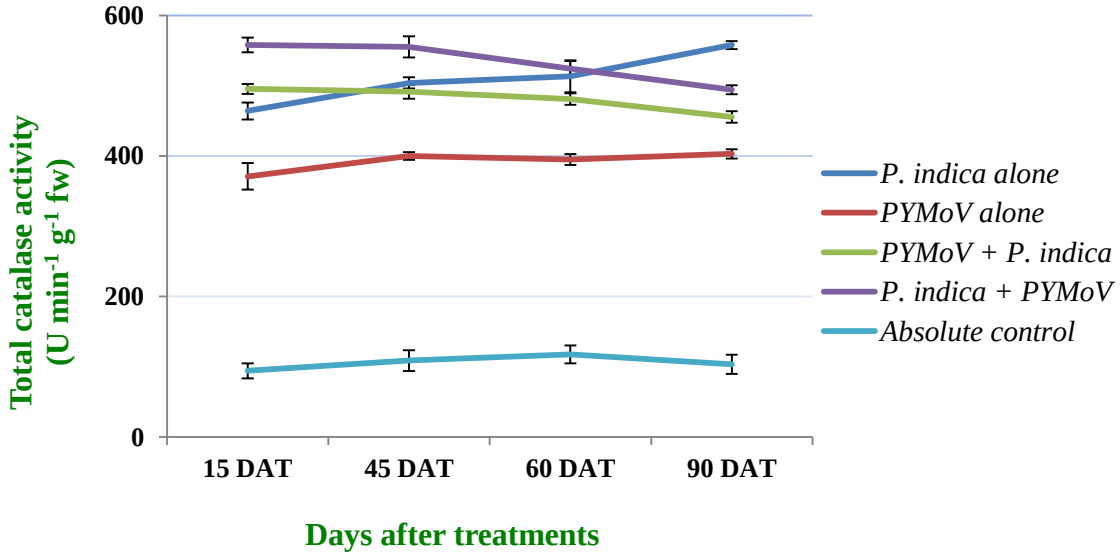


Figure 5: Catalase activity in *P. indica*-colonized black pepper plants (var. Panniyur 1), upon pre- and post-inoculation of PYMoV

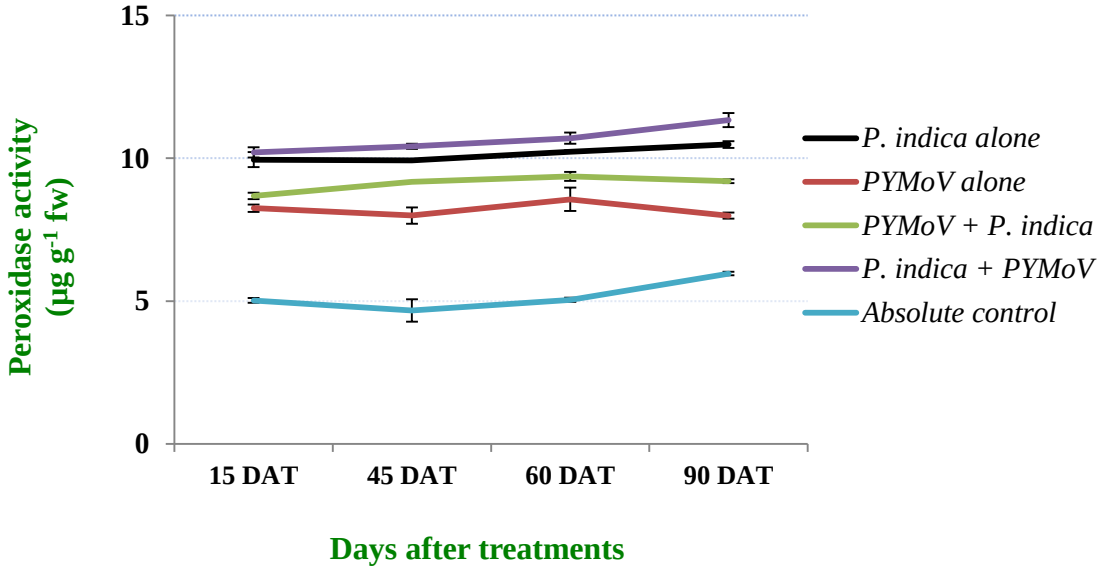


Figure 6: Peroxidase activity in *P. indica*-colonized black pepper plant (var. Panniyur 1), upon pre- and post-inoculation of PYMoV

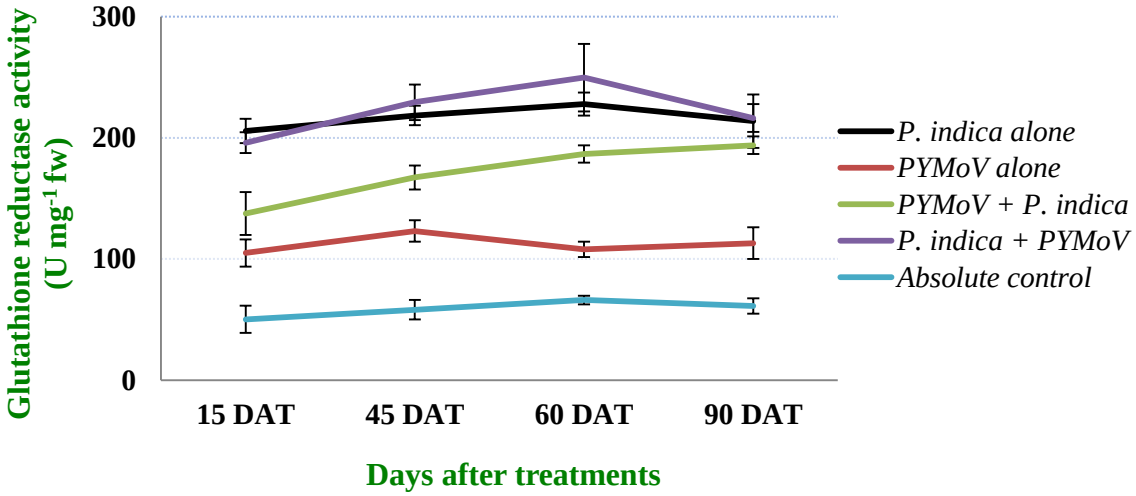


Figure 7: Glutathione reductase activity in *P. indica*-colonized black pepper plants (var. Panniyur 1), upon pre- and post-inoculation of PYMoV

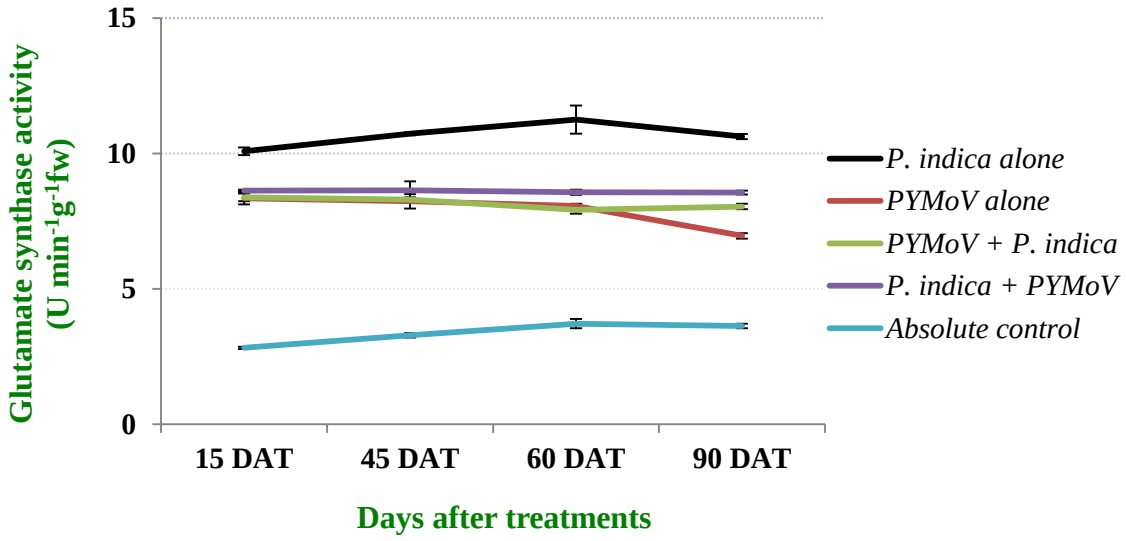


Figure 8: Glutamate synthase activity in *P. indica*-colonized black pepper plants (var. Panniyur 1), upon pre- and post-inoculation of PYMoV

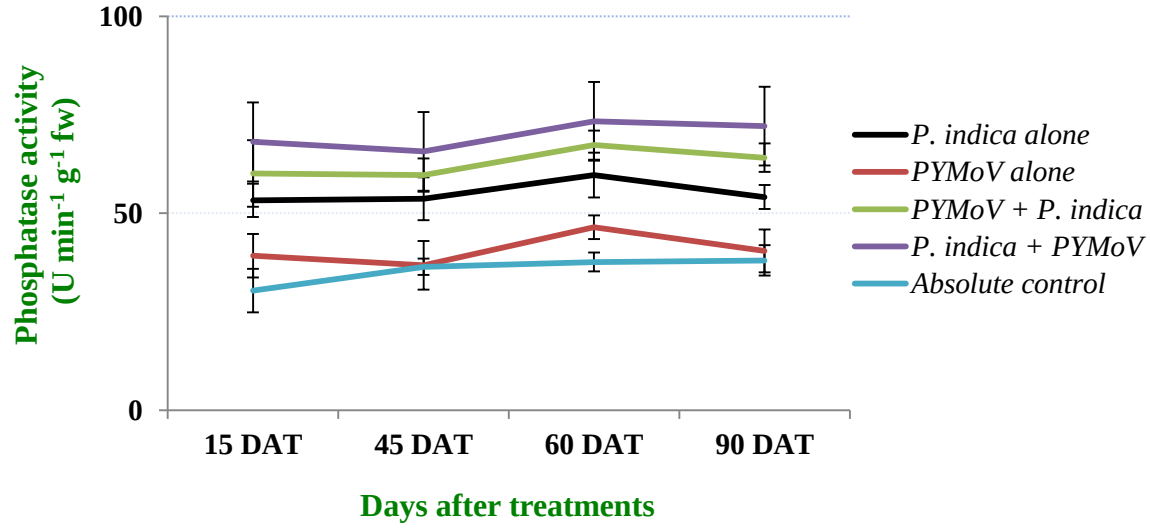


Figure 9: Phosphatase activity in leaves of *P. indica*-colonized black pepper plants (var. Panniyur 1), upon pre- and post-inoculation of PYMoV

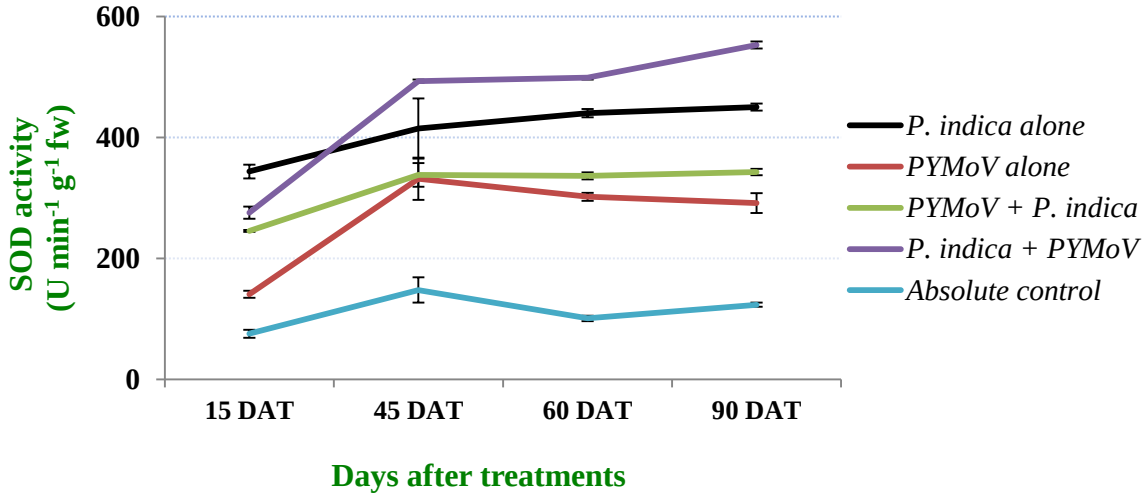


Figure 10: SOD activity in leaves of *P. indica*-colonized black pepper plants (var. Panniyur 1), upon pre- and post-inoculation of PYMoV

Summary

6. SUMMARY

The study entitled "Evaluation of *Piriformospora indica* against *Piper yellow mottle virus* (PYMoV) in black pepper" was conducted in Department of Plant Pathology, College of Agriculture, Vellayani, Thiruvananthapuram during 2019-2021, to evaluate the efficiency of the root colonizing endophytic fungus *P. indica* in managing PYMoV in black pepper, and to elucidate the role of reactive oxygen species and antioxidant enzymes in the tripartite interaction.

A survey conducted in the Instructional Farm of College of Agriculture, Vellayani, to collect and identify PYMoV-infected black pepper plants. Black pepper varieties *viz*., Panniyur 1, Panniyur 2, Panniyur 3, Panniyur 4, Panniyur 5, Panniyur 6, Panniyur 7 and Karimunda were assessed to check their reaction to PYMoV infection revealed that all the above varieties were moderately to highly susceptible to the disease, with an incidence varying from 10 to 91 per cent and the disease severity or intensity assessed as vulnerability index from 30 to 58.

All the surveyed varieties showed almost similar symptoms of PYMoD, like chlorotic flecking, chlorotic mottling, distortion and crinkling of leaves and reduction in leaf area. Under severely infected conditions, varieties like Karimunda and Panniyur 1 even exhibited stunted growth. Leaf tissue from all the collected samples were subjected to PCR using primer pair specific to ORF III of PYMoV. All the samples yielded 400 bp amplicons, thus confirming the presence of the virus.

P. indica culture, obtained from Department of Plant Pathology, College of Agriculture, Vellayani (from Prof. Dr. Ralf Oelmüller, Institute of General Botany and Plant Physiology, Friedrich-Schiller University, Jena, Germany; and originally from Prof. Dr. Ajit Varma; No. INBA3202001787) was sub-cultured in Petri dishes containing PDA, at fortnightly intervals to maintain its virulence. Radial growth of the fungus fully covered nine cm Petri dish at 25 days after inoculation. In PDB, mycelial mat of the fungus was obtained 15 days after inoculation. *P. indica* was mass multiplied in 1:1 coirpith-dried FYM mixture,

amended with two per cent gram flour, where the optimum growth of the fungus was obtained at seven days after inoculation.

Rooted cuttings of black pepper were planted in polybags containing *P. indica*-mass multiplied media, after surface sterilizing the roots. The roots were examined for *P. indica* colonization, every other day, by trypan blue staining. Pear-shaped chlamydospores of the fungus were observed on the surface of the rooted cuttings at seven DAC, and inside the root cortex at 15 DAC. *P. indica* colonization resulted in a significant increase in various biometric parameters of black pepper plants compared to the control plants. *P. indica*-colonized plants showed 25, 23, 35, 23 and 27 per cent more shoot length, root length, number of leaves, leaf area and number of primary roots than the control plants, at 150 days after co-cultivation.

A pot culture experiment was set up to assess the efficacy of *P. indica*, both preventive and curative, in regulating PYMoV of black pepper. When compared to control plants, *P. indica*-colonization significantly reduced the incidence and severity of PYMoD, upon pre- as well as post-inoculation of the virus. Among the pre-inoculation treatments, the best results were obtained when the virus was inoculated onto the plants 30 days after co-cultivation with *P. indica*. Plant height, number of leaves, and leaf area were considerably higher in the *P. indica*-colonized plants upon pre- and post-inoculation of PYMoV compared to the diseased control plants. At 90 days after treatments, the *P. indica*-colonized plants that had been post-inoculated with the virus showed 31 per cent more shoot length, 60 per cent more leaves and 65 per cent more leaf area than the un-colonized diseased plants. Plants pre-inoculated with the virus and then colonized by *P. indica* had 24 per cent more shoot length, 50 per cent more leaves, and 45 per cent more leaf area than the un-colonized control plants upon graft inoculation. Even 90 days after virus inoculation, the *P. indica*-colonized plants that were post-inoculated with the virus showed only mild symptoms such as chlorotic mottling and slight leaf crinkling. *P. indica*-colonization on the plants pre-inoculated with PYMoV resulted in no symptom remission in the previously infected leaves; however, the newly emerging leaves showed only lesser symptoms than the already infected leaves. Un-colonized control plants inoculated with the virus alone, on the other hand, displayed severe

symptoms such as crinkling and deformation of leaves, significant reduction in leaf area and stunted growth. PCR amplification using primer pair specific to ORF III of PYMoV also revealed that the virus titer was lowest in *P. indica*-colonized plants that were post-inoculated with the virus, followed by pre-inoculated plants that were later colonized with *P. indica*, and highest in the un-colonized plants that were inoculated with the virus alone.

To assess the effectiveness of *P. indica* in modulating the biochemical responses of black pepper plants to PYMoV infection, the activity of various enzymes such as catalase, peroxidase, glutathione reductase, glutamate synthase, phosphatase, and superoxide dismutase, which are known to play important roles in the plant antioxidant defense systems, was measured at 15, 45, 60, and 90 days after treatment. The presence of ROS like superoxides and hydrogen peroxide were also detected using nitroblue tetrazolium chloride (NBT) and diaminobenzidine (DAB) stainings respectively. Results showed that *P. indica*-colonization resulted in significant increase in the activity of the above mentioned antioxidant enzymes, upon pre- as well as post-inoculation of PYMoV; which in turn reduced the accumulation of harmful ROS like superoxides and hydrogen peroxide caused due to the virus infection. The same trend was observed throughout the intervals studied, wherein the activity of most of the antioxidant enzymes increased, with the maximum activity observed at 90 DAT and the accumulation of superoxides and hydrogen peroxide was almost negligible at 90 DAT. This might be the reason for better performance of *P. indica*-colonized black pepper plants even after being challenged by PYMoV.

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Appendices

APPENDIX I

Buffers for DNA isolation

CTAB buffer (pH 8)

100 mM Tris-HCl (pH 8)

25 mM EDTA (pH 8)

2 M NaCl

2 % (w/v) CTAB)

TE buffer (pH 8)

10 mM Tris

1 mM EDTA

TAE buffer (50X, pH 8.5)

2 M Tris base

0.5 M EDTA disodium salt

1 M glacial acetic acid

Final volume made up to 1 L using distilled water.

APPENDIX II

Potato Dextrose Agar medium

Potato - 200 g

Dextrose - 20 g

Agar - 20 g

Distilled water - 1000 ml

APPENDIX III

Lactophenol Trypan blue stain

Phenol crystals - 20 g
Lactic acid - 20 ml
Distilled Water - 20 ml
Glycerol - 40 ml
Trypan blue - 0.05 g

APPENDIX IV

Estimation of Total soluble proteins

0.1 M Sodium acetate buffer (pH 4.7)

Solution A - 0.2 M acetic acid (11.5 ml in 1 L distilled water)
Solution B - 0.2 M sodium acetate (16.4 g in 1L distilled water)

22.7 ml of solution A mixed with 27 ml of solution B, and volume made up to 100 mL with distilled water

Stock dye solution (5X)

Coomassie brilliant blue G-250 - 100 mg
95 per cent ethanol - 50 mL
Orthophosphoric acid - 100 mL

Volume made up to 200 mL with distilled water and stored at 4^0C

APPENDIX V

Buffers for the analysis of antioxidant enzymes

0.067 M phosphate buffer (pH 7)

Potassium phosphate monobasic (KH_2PO_4) - 3.52 g

Sodium phosphate dibasic ($Na_2HPO_4.2\ H_2O$) - 7.27 g

Final volume made up to 1 L with distilled water

H_2O_2 - PO_4 buffer

0.16 ml of H_2O_2 (10 % w/v) diluted to 100 ml with phosphate buffer

0.1 M sodium phosphate buffer (pH 6.5)

Solution A : 0.2 M sodium phosphate monobasic (NaH_2PO_4) (27.8 g in 1 L)

Solution B : 0.2 M sodium phosphate dibasic ($Na_2HPO_4.2\ H_2O$) (53.65 g in 1 L)

68.5 mL of solution A mixed with 31.5 mL of solution B and final volume made up to 200 ml with distilled water

0.1 M potassium phosphate buffer (pH 7.5)

Solution A : 1 M potassium phosphate dibasic (K_2HPO_4) (87.09 g in 0.5 L)

Solution B : 1 M potassium phosphate monobasic (KH_2PO_4) (68.04 g in 0.5 L)

80.2 ml of solution A mixed with 19.8 mL of solution B and final volume made up to 1 L

0.05 M sodium citrate buffer (pH 5.3)

Solution A : 0.1 M citric acid (21.01 g in 1 L)

Solution B : 0.1 M sodium citrate (29.41 g in 1 L)

16 ml of solution A mixed with 34 ml of solution B and final volume made up to 100 ml.

Substrate solution for phosphatase estimation (pH 5.3)

EDTA - 1.49 g

Citric acid - 0.84 g

p- nitrophenyl phosphate - 0.03 g

Volume made up to 100 ml with distilled water.

APPENDIX VI

Staining solutions for the detection of ROS

NBT staining solution (0.2 %)

0.1 g NBT dissolved in 0.05 M sodium phosphate buffer (pH 7.5) and volume made up to 50 ml

DAB staining solution (1mg mL^{-1})

50 mg DAB was dissolved in 45 ml distilled water in an amber-coloured bottle. pH adjusted to 3.8 using 0.1 N HCl. Volume made up to 50 ml.

Abstract

Evaluation of *Piriformospora indica* against *Piper yellow mottle virus* in Black pepper

by

LEKSHMI R. KRISHNAN

(2019-11-107)

Abstract of thesis

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ABSTRACT

The study entitled "Evaluation of *Piriformospora indica* against *Piper yellow mottle virus* (PYMoV) in black pepper" was conducted in Department of Plant Pathology, College of Agriculture, Vellayani, Thiruvananthapuram during 2019-2021, to evaluate the efficiency of the root colonizing endophytic fungus *P. indica* in managing PYMoV in black pepper, and to elucidate the role of Reactive Oxygen Species and antioxidant enzymes in the tripartite interaction.

A survey conducted in the instructional farm of College of Agriculture, Vellayani, to identify and collect PYMoV-infected black pepper plants showed that the surveyed varieties *viz.,* Panniyur 1 to 7 and Karimunda were moderately to highly susceptible to PYMoV infection, with the disease incidence ranging from 10 to 91 per cent and disease severity as vulnerability index from 30 to 58. PYMoV-infected black pepper plants showed symptoms like chlorotic flecking, mottling, light necrosis, crinkling and distortion of leaves, reduction in leaf size and stunted growth. Presence of the virus in the samples was confirmed by polymerase chain reaction (PCR) with oligonucleotide primers specific to open reading frame III (ORF III) of PYMoV.

P. indica culture obtained from Department of Plant Pathology was maintained in potato dextrose agar (PDA) and potato dextrose broth (PDB) media. *P. indica* was mass multiplied in coirpith-dried farm yard manure (FYM) mixture (1:1) amended with two per cent gram flour and was allowed to colonize on rooted cuttings of black pepper. *P. indica* colonization enhanced different biometric characters at different intervals, compared to the non-colonized control plants. *P. indica* colonization resulted in 25 per cent more shoot length, 23 per cent more root length, 35 per cent more number of leaves, 23 per cent more leaf area and 27 per cent more number of primary roots than the control plants at 150 days after co-cultivation (DAC).

A pot culture experiment was laid out to evaluate the efficiency of prophylactic as well as curative application of *P. indica,* in managing PYMoV of black pepper. *P. indica*

colonization significantly reduced the incidence and severity of PYMoD, upon pre- as well as post- inoculation of the virus, compared to the control plants. Plants colonized with *P. indica* and later inoculated with PYMoV as well as plants infected by PYMoV and later colonized by *P. indica,* both recovered the loss in biomass caused by the virus infection, compared to the non-colonized plants challenged by the virus. However, prophylactic treatment with *P. indica* resulted in better control of the disease compared to the curative treatment. Amplification of the virus by PCR also revealed that the virus titer was the least in *P. indica*-colonized plants that were post-inoculated with the virus after 30 DAC, followed by pre-inoculated plants that were later colonized with *P. indica*. The virus titer was the highest in the non-colonized plants inoculated with the virus.

In order to evaluate the performance of *P. indica* in modulating the biochemical responses of black pepper plants against PYMoV infection, the activity of various enzymes which are known to play a role in the antioxidant defense systems of plants *viz*. catalase, peroxidase, glutathione reducatse, glutamate synthase, phosphatase and superoxide dismutase were assayed. A significant increase in the activity of these antioxidant enzymes was observed in the *P. indica*-colonized plants compared to the control plants, upon pre- as well as post- inoculation of PYMoV. Thus, the better performance of *P. indica*-colonized plants upon PYMoV infection can be correlated to the increased activities of antioxidant enzymes. PYMoV-infected plants showed increased accumulation of reactive oxygen species like superoxide anions and hydrogen peroxide, which cause oxidative stress in the plants. *P. indica*-colonization was able to mitigate the increase in ROS concentration caused due to PYMoV infection, thus reducing the symptoms.

Thus the study reveals that, co-cultivation of rooted cuttings of black pepper (obtained from disease-free mother plants) with *P. indica* and keeping them under insect-proof conditions for around 30 days (for successful establishment of the fungus inside the root cortex) and then planting them in the main field is a reliable and chemical-free method to control PYMoD in black pepper. Bio-protective action of *P. indica* might be attributed to

the increased activity of antioxidant enzymes, which in turn leads to suppression in ROS accumulation in the colonized plants, upon being challenged by the virus.

സംഗ്രഹം

"*പിരിഫോർമോസ്പോറ ഇൻഡിക്ക* ഉപയോഗിച്ച് കുരുമുളകിലെ *പൈപ്പർ യെല്ലോ മോട്ടിൽ വൈറസിൻറെ* നിയന്ത്രണം" എന്ന വിഷയത്തെ ആസ്പദമാക്കി വെള്ളായണി കാർഷിക കോളേജിലെ സസ്യരോഗ വിഭാഗത്തിൽ 2019 - 2021 കാലയളവിൽ പഠനം നടത്തുകയുണ്ടായി. *പി. ഇൻഡിക്ക* എന്ന വേരിൽ അന്തർവ്യാപനശേഷിയുള്ള മിത്രകുമിളിനു കുരുമുളകിലെ *പൈപ്പർ യെല്ലോ മോട്ടിൽ വൈറസ്* രോഗത്തെ ചെറുക്കാനുള്ള ശേഷി വിലയിരുത്തുക എന്നതും ഇതിൽ വിവിധ റിയാകടീവ് ഓക്സിജൻ ഇനങ്ങൾക്കും നിരോക്സീകരണ എൻസൈമുകൾക്കും ഉള്ള പങ്ക് വിശകലനം ചെയ്യുക എന്നതുമായിരുന്നു പഠനത്തിൻറെ ഉദ്ദേശം.

പഠനത്തിൻറെ ഭാഗമായി വെള്ളായണി കാർഷിക കോളേജിലെ ഇൻസ്ട്രക്ഷണൽ ഫാമിൽ നിന്ന് വൈറസ് രോഗബാധയുള്ള വിവിധ ഇനം കുരുമുളകു തൈകൾ ശേഖരിച്ചു. ഈ കുരുമുളക് ഇനങ്ങളിലെ (പന്നിയൂർ 1, 2, 3, 4, 5, 6, 7, കരിമുണ്ട) രോഗബാധയും രോഗതീവ്രതയും വിലയിരുത്തി. ഏറ്റവും ഉയർന്ന രോഗബാധയും രോഗതീവ്രതയും കരിമുണ്ട ഇനത്തിലാണെന്നു കണ്ടെത്തി. ഇലകളിലെ വിളർച്ചയും മഞ്ഞ നിറത്തിലുള്ള പുള്ളികളുമാണ് ഈ രോഗത്തിൻറെ ആദ്യലക്ഷണം. ഇല ഞരമ്പുകളിലെ വിളർച്ചയെ തുടർന്ന് ഇലകൾ ചുരുളുന്നതായും വളർച്ച മുരടിച്ചു ചുക്കിചുളിഞ്ഞു പോകുന്നതായും വള്ളികളുടെ ഇടമുട്ടുകൾക്ക് നീളം കുറഞ്ഞു വളർച്ച മുരടിക്കുന്നതായും കാണപ്പെട്ടു. രോഗബാധയുള്ള ചെടികളുടെ ഇലകളിൽ നിന്ന് ഡി. എൻ. എ. വേർതിരിച്ചെടുക്കുകയും *പൈപ്പർ യെല്ലോ മോട്ടിൽ വൈറസിൻറെ* പ്രത്യേകമായ പ്രൈമർ ഉപയോഗിച്ച് പി. സി. ആർ. ടെസ്റ്റ് വഴി വൈറസിൻറെ സാന്നിധ്യം പരിശോധിച്ച എല്ലാ ഇനങ്ങളിലും സ്ഥിതീകരിക്കുകയും ചെയ്തു.

ചകിരിച്ചോറും ഉണക്കചാണകപ്പൊടിയും 1:1 അനുപാദത്തിൽ കൂട്ടികലർത്തിയശേഷം രണ്ടു ശതമാനം കടലപ്പൊടി ചേർത്ത മാദ്ധ്യമമാണ് *പി. ഇൻഡിക്ക*യുടെ വംശവർദ്ധനവിനായി ഉപയോഗിച്ചത്. കുരുമുളക് തൈകളുടെ വേരിൽ *പി. ഇൻഡിക്കയുടെ* അന്തർവ്യാപനശേഷിയും വളർച്ചയും വിലയിരുത്തി. വേരിൽ *പി. ഇൻഡിക്കയുടെ* കോളനിവാഴ്ച്ച ചെടിയുടെ വളർച്ചയെ ത്വരിതപ്പെടുത്തുകയും ശാഖാ

വേരുകളുടെ ഉല്പാദനം വർദ്ധിപ്പിക്കുകയും ചെയ്യുന്നതായി കാണാൻ സാധിച്ചു . കൂടാതെ ഇലകളുടെ എണ്ണവും വലുപ്പവും കൂട്ടുകയും ചെയ്തു.

വേരിൽ *പി. ഇൻഡിക്കയുടെ* കോളനിവാഴ്ച്ച കുരുമുളകിലെ *പൈപ്പർ യെല്ലോ മോട്ടിൽ വൈറസ്* രോഗബാധയെ എത്രത്തോളവും എങ്ങനെയും തടയുന്നു എന്നതിനെക്കുറിച്ചും പഠനം നടത്തി. വൈറസ് രോഗബാധയ്ക്കു മുൻപുള്ള *പി. ഇൻഡിക്കയുടെ* കോളനിവാഴ്ച്ചയും രോഗബാധയ്ക്കു ശേഷമുള്ള കോളനിവാഴ്ച്ചയും വൈറസ് ബാധയെ ചെറുക്കാൻ കുരുമുളക് തൈകളെ സഹായിക്കുന്നതായി കാണാൻ സാധിച്ചു; എങ്കിലും വൈറസ് രോഗബാധയ്ക്കു മുൻപുള്ള *പി. ഇൻഡിക്കയുടെ* കോളനിവാഴ്ച്ച ഈ രോഗത്തെ കൂടുതൽ ഫലപ്രദമായി പ്രതിരോധിക്കും എന്നും കണ്ടെത്തി. *പി. ഇൻഡിക്ക* ഇല്ലാത്തതും രോഗം ബാധിച്ചതുമായ ചെടികളെക്കാൾ ഹരിതകം, പ്രോട്ടീൻ, നിരോക്സീകരണ എൻസൈമുകളായ കാറ്റലേസ്, പെറോക്സിഡേസ്, ഫോസ്ഫറ്റേസ്, ഗ്ലൂട്ടാതയോൺ റിഡക്റ്റേസ്, ഗ്ലൂട്ടാമേറ്റ് സിന്തേസ്, സുപ്പറോക്സയിട് ഡിസ്മ്യൂട്ടേസ് എന്നിവയുടെ പ്രവർത്തനം *പി. ഇൻഡിക്ക* കോളനിവാഴ്ച്ചയുള്ള ചെടികളിൽ ത്വരിതപ്പെടുത്തുന്നതായും കാണപ്പെട്ടു.

വേരുപിടിച്ച കുരുമുളകു തൈകളിൽ *പി. ഇൻഡിക്ക* കോളണൈസ് ചെയ്യിച്ചു മുപ്പതു ദിവസത്തോളം രോഗവാഹികളായ കീടങ്ങളിൽനിന്നു സംരക്ഷണം നൽകിയശേഷം അവയെ കൃഷിയിടത്തിൽ നടുന്നത് *പൈപ്പർ യെല്ലോ മോട്ടിൽ വൈറസ്* ബാധയെ നല്ലരീതിയിൽ നിയന്ത്രിക്കാൻ സഹായിക്കും എന്ന് ഈ പഠനം കൊണ്ട് വ്യക്തമാകുന്നു.