Management of *Bhendi yellow vein mosaic virus* using beneficial fungal root endophyte *Piriformospora indica*

CHIPPY

(2018-11-011)

DEPARTMENT OF PLANT PATHOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695522

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by

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THESIS

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2020

DECLARATION

I, hereby declare that this thesis entitled **"Management of *Bhendi yellow vein mosaic virus* using beneficial fungal root endophyte *Piriformospora indica*"** 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.

Vellayani **Chippy**

Date: 19.10.2020 (2018-11-011)

CERTIFICATE

Certified that this thesis entitled **"Management of *Bhendi yellow vein mosaic virus* using beneficial fungal root endophyte *Piriformospora indica*"** is a record of research work done independently by Ms. Chippy under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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We, the undersigned members of the advisory committee of Ms. Chippy, a candidate for the degree of **Master of Science in Agriculture** with major in Plant Pathology, agree that the thesis entitled **"Management of *Bhendi yellow vein mosaic virus* using beneficial fungal root endophyte *Piriformospora indica*"** may be submitted by Ms. Chippy, in partial fulfilment of the requirement for the degree.

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Chippy

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LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Per cent
µg	Microgram
µL	Microlitre
µm	Micrometer
^{o}C	Degree Celsius
BYVMV	*Bhendi yellow vein mosaic virus*
BYVMD	Bhendi yellow vein mosaic disease
AMF	Arbuscular mycorrhizal fungi
BlCMV	*Blackeye cowpea mosaic virus*
CD	Critical difference
CRD	Completely Randomized Design
Cm	Centimeter
DAI	Days after inoculation
DAC	Days after co-cultivation
et al	And other co workers
G	Gram
H	Hour
KM	Kafer medium
L	Litre
ELISA	Enzyme Linked Immunosorbent Assay
MS medium	Murashige and Skoog medium
M	Molar
mM	Milli molar
ml	Millilitre
mg	Milligram
mm	Milli meter
min.	Minute
nm	Nano meter
OD	Optical density
PDA	Potato dextrose agar

PDB	Potato dextrose broth
PO	Peroxidase
PPO	Polyphenol oxidase
CAT	Catalase
PR-protein	Pathogenesis related protein
PVP	Poly vinyl pyrrolidone
PNM	Plant nutrient medium
Rpm	Rotations per minute
SDM	Spray dried milk
SE (m) $\pm$	Standard error of mean
WAC	Week after co-cultivation
viz.,	Namely
D. I.	Disease incidence
V. I.	Vulnerability index
DNA	Deoxyribo nucleic acid
CTAB	Cetyl trimethyl ammonium bromide
ROS	Reactive oxygen species
PCR	Polymerase chain reaction
GR	Glutathione reductase
SOD	Superoxide dismutase
PAL	Phenylalanine ammonia lyase
ZYVMV	*Zucchini yellow vein mosaic virus*
PVY	*Potato virus Y*

Introduction

1. INTRODUCTION

Okra (*Abelmoschous esculentus* L. Moench) also called as bhendi or lady's finger belonging to the family Malvaceae, is one of the most important vegetable crops grown in tropical, subtropical and warmer temperate regions around the world. Being the native of tropical Africa (Purseglove, 1987), okra is one among the most heat- and drought-tolerant vegetable species in the world. India is the largest producer of okra covering an area of 5.13 lakh ha with an annual production of 61.70 lakh tones (NHB, 2018). The major okra producing states are Uttar Pradesh, Assam, Bihar, Orissa, Maharashtra, West Bengal and Karnataka.

Okra is utilized for its edible tender green fruits, that are either cooked, pickled, eaten raw, or included in salads (Gemede *et al.,* 2016); and also valued for its nutritional qualities, particularly high content of carbohydrates, protein, calcium, iron and vitamin C (Arapitsas, 2008) and moderate contents of thiamine, folate and magnesium, thus plays a vital role in human diet. It has a wide spectrum of uses including seeds used as substitute for coffee; bast fibre for the reinforcement of polymer composites (De Rosa *et al*., 2010); mucilage for the removal of turbidity from wastewater; dry fruit skin and fibres in manufacture of paper, card board and fibres; and dried fruits that contain 13-22 per cent edible oil used for production of refined edible oil (Anastasakis *et al.,* 2009).

A number of fungi, bacteria, viruses, nematodes and pests attack this crop, leading to heavy crop loss. At least 19 different plant viruses infect okra; yellow vein mosaic, enation leaf curl and okra leaf curl are the common viral diseases of okra resulting upto 100 per cent crop loss (Swanson and Harrison, 1993; Venkataravanappa *et al.,* 2013). Among the viral diseases, yellow vein mosaic disease is the most devastating disease in Indian subcontinent that causes major constraint in production resulting in huge losses to farmers (Prakasha *et al.,* 2010).

Bhendi yellow vein mosaic disease was first reported by Kulakarni (1924) from Bombay presidency, India but the causal agent as a virus was confirmed by Uppal *et al.,* (1940) and suggested the name “yellow vein mosaic”. BYVMV belongs to the genus *Begomovirus* in the family *Geminiviridae*, has a bipartite genome consisting of two ssDNA molecules of 2.6 - 2.8 kb DNA-A, that helps in

encapsidation and replication; DNA-B, involved in systemic movement of virus and an associated beta satellite that depend on DNA-A component for encapsidation, replication, and insect transmission (Jose and Usha, 2003; Venkataravanappa *et al.*, 2012).

Characteristic symptoms of the disease include vein clearing, severe infection leading to complete yellowing of leaves, reduction in leaf size and stunting of plants. Symptoms on fruits include malformation, hardening and bleaching (Prakasha *et al.*, 2010). The yield loss due to BYVMV ranges from 50 to 90 per cent depending on stage of crop (Sastry and Singh, 1974). The virus is transmitted by whitefly (*Bemisia tabaci)* in a circulative non-propagative manner. A number of weeds viz., *Ageratum conyzoides, Croton sparsiflorous, Malvastrum tricuspidatum etc.* act as collateral hosts of BYVMV which serves as source of inoculum, thus making its management difficult (Naveen, 2018).

Viral diseases cause huge losses in production and quality of crops owing to its obligate nature; thus becomes a serious threat to global food security (Varma and Malathi, 2003). The viral diseases are very difficult to control once infected. In severe cases, complete destruction of infected plants becomes the only alternative for its management. Current management strategy of BYVMV includes use of resistant or tolerant varieties, control of vectors through cultural or chemical methods. Use of beneficial fungal endophytes is a novel, durable, sustainable and ecofriendly alternative for management viral diseases that act as bioprotectors, bioregulators, biofertilizers and growth promoters.

Beneficial micro-organisms including root endophytes form symbiosis with plants and play an important role in maintaining a better soil and plant health (Smith and Smith, 2011). *Piriformospora indica*, an axenically cultivable phytopromotional, biotrophic mutualistic root endophytic fungus that belongs to the order Sebacinales under class Basidiomycota, isolated from xerophytic shrubs of Thar desert in India by Verma in 1998, is utilized for growth promotion, abiotic (drought, water, cold, salinity, high temperature and heavy metals) and biotic (root and foliar pathogens, nematodes) stress management in crop plants (Varma *et al.*, 1999). Moreover, *P. indica* colonization induces early flowering and enhance seed production, and helps in

the hardening of tissue-cultured plants (Oelmuller *et al*., 2009; Varma *et al*., 2012a, b; Johnson *et al*., 2014; Gill *et al*., 2016).

P. indica is widely exploited for the management of many root and foliar fungal and bacterial diseases (Johnson *et al*., 2014). Less number studies were undertaken on the exploitation of *P. indica* in the management of viral diseases in crop plants, particularly DNA viruses. Recently it has been reported that *P. indica* primed plants showed enhanced resistance against *Tomato yellow leaf curl virus* (Wang *et al.*, 2015*), Cowpea aphid borne mosaic virus* (Alex, 2017) and *Cowpea blackeye mosaic virus* (Chandran, 2019).

In this context, the present study was undertaken with the following objectives,

1. To survey and study the symptomatology, and molecular detection of BYVMV infected okra samples in Thiruvananthapuram.
2. To evaluate *P. indica*-primed okra seedlings for management of BYVMV.
3. To elucidate role of oxidative and ROS scavenging enzymes involved in *P. indica* mediated resistance.

Review of Literature

2. REVIEW OF LITERATURE

Okra also known as bhendi or lady's finger is a multipurpose herbaceous annual plant, belonging to Malvaceae family, mainly cultivated for its edible tender fruits. Among various viral diseases affecting the crop, bhendi yellow vein mosaic disease is the most destructive one which results in immense crop loss and serious deprivation in market value (Uppal *et al.,* 1940; Capoor and Varma, 1950). Once systemically infected, the virus is very difficult to manage owing to its obligate nature and unique transmission mode as in case of any other viral disease. A very promising alternative is the use of beneficial endophytic microbes for the management of the viral disease in addition to the enhanced plant growth.

2.1. COLLECTION OF BYVMV INFECTED OKRA PLANTS

Prashanth *et al*. (2008) screened fifty-five genotypes of okra against yellow vein mosaic under field conditions and reported that the per cent disease incidence and coefficient of infection ranged from 7.2 to 100.0 and 1.8 to 75.0 respectively. Venkataravanappa (2008) conducted survey on Geminivirus associated with okra in India and reported that bhendi yellow vein mosaic disease (BYVMD) incidence ranged from 42.45 - 75.64 per cent in Kerala, 23.00 - 75.64 per cent in Tamil Nadu, 23.00 - 67.67 per cent in Karnataka, 45.89 - 56.78 per cent in Andhra Pradesh. Prakasha *et al*. (2010) carried out the survey in okra fields of Dharwad, Haveri, Belgaum and Tumkur districts of Karnataka and reported disease incidence ranging from 0.5 - 48.9 per cent. The survey carried out by Sheikh *et al.* (2013) in Aligarh for incidence of bhendi yellow vein mosaic disease in different seasons revealed the disease incidence ranging from 1.5 to 19.5 per cent in rabi and 23.5 to 36.8 per cent in kharif season.

Ghevariya and Mahatma (2017) conducted survey in Navsari districts of Gujarat and reported a 10 - 50 per cent disease incidence and all the surveyed fields were severely infected with the okra yellow vein mosaic disease. Senevirathna *et al.* (2016) reported 90 per cent incidence of BYVMD from Sri Lanka where plants exhibited characteristic symptoms of vein clearing, reduction in leaf size, stunted growth, and bleaching and malformation of fruits. Naveen (2018) reported highest

disease incidence of 100 per cent in Chirayinkeezhu taluk in Thiruvanavthapuram district of Kerala with vulnerability index (V. I.) of 78.42. Survey conducted in Thiruvananthapuram and Kollam districts of Kerala during 2018 revealed high disease incidence (D. I.) in all surveyed locations with high vulnerability index of 86.6 in Mukhathala taluk of Kollam (Basheer, 2019).

2.2. SYMPTOMATOLOGY STUDIES

The characteristic symptoms of BYVMD include vein clearing and vein thickening of infected leaves. Severe infection of *Bhendi yellow vein mosaic virus* (BYVMV) results in vein swelling, downward curling of leaf margin and twisting of petioles (Capoor and Varma, 1950). Okra plants infected by BYVMV expressed persistent symptoms of vein clearing which later turned into complete chlorosis (Pun and Doraiswamy, 1999). Reduction of leaves and fruit size was also reported (Bhagat *et al.,* 2001). Jose and Usha (2003) reported vein twisting, petiole bending, upward curling and stunted plant growth, in addition to vein clearing and complete yellowing of leaves. Infected plants produce very few deformed leaves and fruits of reduced size, that become yellowish or creamy colour. Virus infection at early stages of crop leads to failure in flowering and fruit production, fruits if formed are small in size, hardened, bleached and deformed (Bhagat *et al.,* 2001; Kucharek, 2004; Taware *et al*., 2010; Senevirathna *et al.,* 2016).

2.3. MOLECULAR DETECTION OF BYVMV

Ghosh *et al.* (2009) modified Cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990) of DNA isolation by including use of more volume of extraction buffer and 1M NaCl, making it suitable for PCR-based detection of begomoviruses from mucilaginous plants. CTAB method was used for total DNA isolation from the infected okra (Venkataravanappa *et al.,* 2015).

Presence of a begomovirus component equivalent to DNA-A was identified in yellow vein infected bhendi plants by PCR-amplification using begomovirus-specific primers (Jose and Usha, 2000). PCR was done using BYVMV specific coat protein primers designed by Jose and Usha (2000) for the detection of viral DNA in plant growth promoting rhizobacteria treated bhendi plants and revealed a reduction in viral

inoculum load in the treated plants (Patil *et al.,* 2011). Viral DNA was isolated from BYVMV infected okra plants by CTAB method and PCR amplification of DNA-A molecule was done using the virus specific primers. The sequenced virus showed highest similarity (98%) with *Okra yellow vein mosaic virus* (Aurangabad) segment DNA-A (Ghevariya and Mahatma, 2017). Biswas *et al.* (2018) reported that DNA-A sequences of whitefly transmitted begomoviruses infecting okra in India are extensively diverse. Naveen (2018) reported PCR based molecular detection of BYVMV using Deng and AV/AC primers specific to coat protein (CP) of *Begomovirus* and showed that the virus has more than 90 per cent similarity to *Okra enation leaf curl virus.* The complete sequence of CP gene of the different BYVMV isolates revealed maximum similarity of about 99 per cent to *Okra enation leaf curl virus* (Basheer, 2019). Biswas *et al.* (2018) reported that DNA-A sequences of whitefly transmitted begomoviruses infecting okra in India are extensively diverse.

2.4. CO-CULTIVATION OF OKRA WITH BENEFICIAL ROOT ENDOPHYTIC FUNGUS, *Piriformospora indica*

2.4.1. Maintenance of *P. indica* culture

Various synthetic and complex medium can be utilized for culturing and maintenance of *P. indica*. Sahay and Varma (1999) could grow culture of *P. indica* in modified minimal agar medium at pH 4.8. Rhythmic growth pattern was observed when *P. indica* was cultured on modified Aspergillus minimal medium at 30^{o}C for 8 days in dark (Peskan-Berghofer *et al.,* 2004; Pham *et al.,* 2004). *P. indica* culture was maintained on Aspergillus minimal medium (Hill and Kaefer, 2001; Druege *et al*., 2007). *P. indica* was grown in modified Kaefer medium in a temperature- and light-controlled growth chamber (Sun *et al.*, 2010). Optimum growth and sporulation of *P. indica* was achieved when peptone, yeast extract and soya bone meal was added to modified Kaefer medium at a concentration of 2.0 g L^{-1} each (Kumar *et al.,* 2011). Maximum growth of *P. indica* was obtained in PDA as well as Kaefer medium among various media tested for cultivation of *P. indica* (Kumar *et al.,* 2012a). Optimum chlamydospore production was obtained when *P. indica* was grown on 4 per cent (w/v) of jaggery (Kumar *et al.,* 2012b). *P. indica* was maintained by transferring four weeks-old culture into fresh modified Kaefer medium and incubating in dark at

22 - 24°C (Johnson *et al*., 2013). *P. indica* was cultured on Kaefer medium and incubated at 28°C for 10 days (Tarte *et al.,* 2019; Khalid *et al*., 2020).

Tanha *et al.* (2014) maintained *P. indica* culture in CM (complex medium) and incubated at 25^{o}C for one month to allow enough sporulation. Maximum mycelial growth and chlamydospore production were observed in four per cent jaggery medium, and in medium containing nitrogen, yeast extract and peptone (Varma *et al*., 2014).

P. indica was cultured on Hill and Kaefer medium solidified with 1 per cent agar by incubating in dark at 28 ± 2°C for 7 days (Prasad *et al.,* 2013; Kilam *et al*., 2017). Nivedita *et al.* (2017) maintained *P. indica* culture on modified solid Aspergillus at 30°C in dark. *P. indica* was cultured on Aspergillus modified medium standardised by Hill and Kaefer, (2001) and incubated at 30 ± 2 °C for 7-10 days (Narayan *et al.,* 2017). The culture of *P. indica* was maintained on potato dextrose agar (PDA) medium at room temperature (28 ± 2°C) in dark (Fakhro *et al*. 2010; Sharma *et al.,* 2014; Lakshmipriya, 2017; Nassimi and Taheri, 2017; Anith *et al.,* 2018; Cheng *et al*., 2020).

Liquid media can be used for maintenance and mass cultivation *P. indica*. Varma *et al.* (2012a) optimized the growth conditions of *P. indica* in modified liquid Hill–Kaefer medium at pH 6.5 and temperature 30^{o}C with an agitation speed of 200 rpm. Maximum dry cell weight and spore yield was obtained at 5 days after inoculation. *P. indica* was grown in Kaefer liquid media with an optimal pH of 6.5 (Hill and Kafer 2001) and incubated at 28°C with 200 rpm agitation speed (Kumar *et al.,* 2016; Sadiqque, *et al.,* 2018). Liquid culture of *P. indica* was obtained by placing 2-3 fungal mycelial bits in 100 ml potato dextrose broth (PDB) medium and maintained at temperature of 28°C in the dark for 3 days at 200 rpm agitation speed (Cheng *et al*., 2020).

2.4.2. Co-cultivation of *P. indica* with okra

For establishment of co-cultivation of *P. indica* with barley, two days-old barley seedling roots were immersed in an aqueous solution of 0.05 per cent Tween 20 containing 5 x 10^{5} ml^{-1} *P. indica* chlamydospores and grown in mixture of expanded

clays (Deshmukh *et al.*, 2007). Co-cultivation of *Linum album* cell suspension culture with *P. indica* was done in Gamborg's B5 media inoculated with different concentrations of the five days-old fungal cultures resulted in phytopromotional effects (Baldi *et al.,* 2008; Kumar *et al*., 2012a). The co-culture of *P. indica* with maize plants was established by growing plants in sterile soil inoculated with one per cent fungal mycelium that was mixed with Hoagland's solution (Kumar *et al*., 2009). Achatz *et al*. (2010) performed co-cultivation of *P. indica* with barley plants by growing plants in 300 g sterile substrate mixed with 2 g of fungal mycelium.

For colonization in *Coleus forskohlii*, *P. indica* mycelium in liquid Hill and Kafer medium was filtered, washed and placed in potting mixture as sandwich layer model (Das *et al*., 2012). Media containing MS and PDA in 1:1 ratio was used for effective co-cutivation of *P. indica* with *Centella asiatica* (Satheesan *et al*., 2012). Johnson *et al*. (2013) standardised co-cultivation protocols for *P. indica* with the model plant *Arabidopsis thaliana. In vitro* co-cultivation was established by transferring nine to twelve days-old Arabidopsis seedling and 4 weeks-old *P. indica* plugs to modified PNM medium simultaneously. These seedlings after two weeks were transferred to sterile soil-vermiculite mix for *in-vivo* co-cultivation. Two weeks-old tomato seedling roots were immersed in 10^6 ml^{-1} of *P. indica* chlamydospores suspension for 12 h, transferred into pots containing sterilized mixture of sand and perlite substrate; and its cocultivation resulted in increased antioxidant production in tomato (Sartipnia *et al*., 2013).

For *in vitro* co-cultivation with *P. indica*, surface sterilized *Lotus japonicus* and *Arabidopsis thaliana* seedlings germinated on modified solid Hoagland's medium treated with 1 ml of 0.002 per cent Tween-20 suspension containing 5×10^5 *P. indica* chlamydospores were done; and the *in vivo* co-culture was obtained by growing plants in sterile substrate inoculated with 1 per cent fungal mycelium (Banhara *et al.,* 2015). *Artemisia annua* callus treated with *P. indica* culture filtrate could effectively increase its biomass (Baishya *et al.,* 2015). Royalwar *et al*., (2015) cultured *P. indica* in liquid malt extract medium incubated at 25 - 28^{o}C for 15 days at 100 rpm; and the co-cultivation of *P. indica* with tomato was obtained by transplanting 10 days-old tomato seedlings into sterile soil mixed with 2 per cent fungal mycelium.

Modified Kaefer medium was used for the propagation of *P. indica* for 14 days; mycelium bits from this was transferred to PNM media and kept for fungal growth (Johnson *et al*., 2013). Four 12 days-old seedlings of *A. thaliana* that were grown on MS media were transferred to the PNM plates containing *P. indica* (Vahabi *et al*., 2016). *P. indica* was grown on a complex medium (CM) at 24^{o}C; barley seedling roots were immersed in an aqueous solution of 0.02 per cent Tween-20 containing 5×10^{5} spores ml^{-1} and transferred to substrate 2:1 mixture of sand and perlite for co-cultivation (Ghaffari *et al*., 2016).

Arunkumar and Shivaprakash, (2017) cultured *P. indica* in Hill and Kaefer broth medium and 20 g of fungal mycelium was inoculated in potting mixture following the sandwich layer model for colonization in finger millet. Two weeks-old rice seedlings were dipped in chlamydospores suspension of *P. indica* and transferred to pots filled with a 1:1 (V : V) mixture of sterile sand and soil for co-cultivation (Nassimi and Taheri, 2017). Co-cultivation experiment with pigeon pea was done by inoculating the fungal mycelium that was grown in Kaefer broth medium, near the roots of two weeks-old pigeon pea seedlings grown in sterilized mixture of sand and soil in the ratio 3:1 (Rajak *et al.,* 2017). Su *et al*. (2017) did *in vitro* co-cultivation of *Brassica napus* with *P. indica* by transferring two days-old seedling to modified PNM medium inoculated with *P. indica* and after 15 days of co-culture, the seedlings were transplanted into pots filled with 4:2:1 mixture of sphagnum : vermiculite : perlite.

Establishment of co-culture with black pepper plants was done by mixing one per cent *P. indica* mycelial mass that was grown in PDB, with sterile vermiculite and 50 g of this inoculum was added into a small cavity made in the potting medium and the rooted black pepper cuttings was then planted in the cavity above the inoculum (Anith *et al*., 2018). *P. indica* was grown in Kaefer liquid media (Hill and Kafer, 2001) at a pH of 6.5 at 28°C and shaken at 130 rpm. For colonization, surface sterilized rice seeds were coated with a paste of *P. indica* in vermiculite and colonisation was observed after 4 days of germination (Sadiqque *et al.,* 2018).

Surface sterilized seeds of *Solanum melongena*, *Abelmoschus esculentus* and *Capsicum annuum* were transferred to medium containing MS and PDB (containing *P. indica*) in a 1: 1 ratio, for co-cultivation with *P. indica* (Jisha *et al.,* 2019). For

co-cultivation of *P. indica* with banana, three to four leaved banana plantlets were transferred to rooting medium mixed with *P. indica* chlamydospore suspension that was added before the medium solidified (Li *et al.,* 2019). *P. indica* suspension containing about 60 g mycelia mass L^{-1} and 1×10^5 chlamydospores ml^{-1} was directly poured onto the soil close to root system of one-month old banana plantlets, at concentration of 100 ml kg^{-1} soil (Cheng *et al*., 2020).

2.4.3. Root colonization efficiency of *P. indica*

P. indica colonization increased with root tissue maturation in barley and maximum colonization was in differentiation zone characterized by presence of inter- and intracellular hyphae and intracellular chlamydospores (Deshmukh *et al.,* 2006). *P. indica* formed intracellular hyphae in epidermal cells of wheat after one week of inoculation and chlamydospores within epidermal and root hair cells after 3 weeks of inoculation (Serfling *et al*., 2007). Maize plants showed 20 - 30 per cent colonization at 10^{th} day and colonization increased up to 70 per cent at 20 days after inoculation (DAI) with *P. indica* (Kumar *et al.,* 2009). Colonization pattern of *P. indica* in barley roots was assessed. Inter and intra-cellular hyphae was observed in the rhizodermis and cortex of barley roots and the fungal sporulation initiated at 14 DAI (Schafer *et al.,* 2009).

Turmeric roots treated with *P. indica* showed inter- and intra- cellular root colonization with intra-cellular chlamydospores and the colonization ranged from 60 - 70 per cent (Bajaj *et al.,* 2013). Dong *et al.* (2013) demonstrated that *P. indica* colonization in Chinese cabbage led to the early root maturation with increase in growth and biomass. *Aloe vera* plants co-cultured with *P. indica* showed 67.5 per cent colonization and inoculated plants had higher gel and aloin content (Sharma *et al.,* 2014). Das *et al.* (2014) studied interaction of *P. indica* with *Coleus forskohlii* and showed 25.55 per cent root colonization. *P. indica* co-cultured with globe artichoke showed high degree of the root colonization of 90 per cent and increase in growth parameters under water stress (Tanha *et al.,* 2014).

In vitro-grown plantlets of *Stevia rebundiana* with *P. indica* showed root colonization ranging from 50.0 to 53.3 per cent; while the greenhouse grown plants showed 56.7 to 63.3 per cent (Kilam *et al.,* 2017). Su *et al.* (2017) studied effect of *P.*

indica colonization in *Brassica napus* and observed increase in lateral branching and root hair.

Anthurium plants were colonized by *P. indica* at 14 DAI; the fungal hyphae passed through the root epidermal cellular layers and multiplied in the cortex layers, producing large amounts of spores (Lin *et al*., 2019). In groundnut, fully developed intra-cellular pear shaped chlamydospores in single, double or tetrad chain was observed after 45 days of co-culture with a root colonization ranging from 50 - 60 per cent (Tarte *et al*., 2019).

2.5. EVALUATION OF *P. indica*-PRIMED OKRA SEEDLINGS AND PLANTS AGAINST BYVMV

Only few studies highlighted a protective role of the *P. indica* colonisation against viral infection. First report of effect of *P. indica* against viral disease was by Fakhro *et al*. (2010) where *P. indica* colonisation repressed the amount of *Pepino mosaic virus* in tomato plants. *P. indica*-primed tomato plants showed reduced disease incidence and severity against *Tomato yellow leaf curl virus* by 26 per cent and 1.25 per cent respectively in susceptible cultivar by upregulating expression levels of pathogenesis related genes including *PAL, PR1a, PR3a, PR3b* and *PR5* that are mediated by salicylic acid signalling pathway (Wang *et al*., 2015). Colonisation by *P. indica* reduced the vulnerability index by increased activity of defense enzymes like peroxidase, polyphenol oxidase and by induction of pathogenesis related proteins against *Black eye cowpea mosaic virus* (BlCMV) in cowpea (Alex, 2017; Chandran, 2019). *P. indica*-root colonization in local lesion host *Chenopodium amaranticolor* significantly reduced the local lesions developed by BlCMV with the per cent inhibition of 60 - 73 over control (Chandran, 2019).

Colonization of Arbuscular Mycorrhizal Fungus (AMF), *Rhizophagus irregularis*, in tobacco and cucumber plants followed by *Tobacco mosaic virus* (TMV) and *Cucumber green mottle mosaic virus* (CGMMV) infection respectively reduced the disease symptoms and virus titre (Stolyarchuk *et al*., 2009). AMF, *Funneliformis mosseae* colonization led a beneficial effect on tomato plants by reducing disease severity index (DSI) and showed lower shoot and root concentrations of viral DNA in mycorrhiza colonised virus inoculated plants than in virus inoculated plants (Maffei *et*

al., 2014). Thiem *et al*. (2014) investigated the effect of mycorrhizal colonization on potato plants already infected by *Potato virus Y* (PVY). Milder symptoms and stimulation of shoot growth were observed in the PVY - infected plants inoculated with *R. irregularis.* AMF (*Funneliformis mosseae, Rhizophagus aggregatus* and *Rhizoglomus clarum*) colonization of *Tomato mosaic virus* (ToMV) - infected tomato plants significantly reduced both disease severity and incidence compared to the non–mycorrhizal ToMV- infected plants by triggering the expression of flavonoid and chlorogenic acid biosynthetic pathway genes. Moreover, there was a decreased transcriptional expression level of ToMV-coat protein gene (Aseel *et al*., 2019). *Neotyphodium uncinatum* colonised plants produced alkaloids (lolines) that deterred aphid vectors (Schardl and Philips, 1997; Lehtonen *et al.,* 2005); thus, reduced the infection of *Barley yellow dwarf virus* (Lehtonen *et al*., 2006). Endophytic fungus *Beauveria bassiana* could systemically colonise the entire plant through intercellular spaces and vascular xylem elements; and inhibited the systemic movement of *Zucchini yellow vein mosaic virus* (ZYVMV) from cell to cell (Landa *et al.,* 2013) thus resulted in decreased disease incidence and severity of ZYVMV in squash plants (Jaber and Salem, 2014). Muvea *et al*. (2015) reported that colonization of the onion plant by fungus *Hypocrea lixi* reduced feeding, oviposition and survival of onion thrips, *Thrips tabaci* and inhibited the replication of *Iris yellow spot virus (Tospovirus).* There was a 2-fold reduction in feeding activity of viruliferous *T. tabaci* in *Hypocrea lixi*-colonized onion plants (Muvea *et al.,* 2018).

Expression levels defense related genes *viz., PR-1a*, phenylalanine ammonia-lyase *(PAL*), and 3-hydroxy-3-methylglutaryl CoA reductase (*HMGR*) were upregulated in *Bacillus amyloliquefaciens* (strain EXTN-1) treated tobacco plants; thus, inhibited *Pepper mild mottle virus* (Ahn *et al.,* 2002). *Bacillus amyloliquefaciens,* a leaf colonizing bacteria, conferred protection against mechanically transmitted *Cucumber mosaic virus* (CMV) and natural incidence of *Broadbean wilt virus* and *Pepper mottle virus* by enhancing salicylic acid and jasmonic acid defense signalling pathways in infected chilli plants (Lee and Ryu, 2016). Lee *et al.* (2017) found that foliar application of the leaf colonizing yeast *Pseudozyma churashimaensis* elicited defense pathways and conferred protection

against CMV, *Pepper mild mottle virus, Pepper mottle virus, and Broad bean wilt virus* under field conditions.

Raupach *et al.* (1996) showed that seed treatment with plant growth-promoting rhizobacteria (PGPR) strains (*Pseudomonas fluorescens* and *Serratia marcescens*) induced systemic resistance against CMV infection in cucumber and tomato plants. *P. fluorescens* strain CHA0 showed induced resistance against *Tobacco necrotic virus* (TNV) in tobacco (Maurhofer *et al.,* 1994). Murphy *et al.* (2000) reported that tomato plants treated with PGPR showed reduced symptom and incidence of *Tomato mottle virus.* PGPR treatment induced resistance against CMV in tomato plants (Zehnder *et al.,* 2000). Studies conducted by Kandan *et al.* (2002) showed that application of *P. fluorescens* (CoP-1 and CoT-1 and CHAO) reduced *Tomato spotted wilt virus* (TSWV) infection by 84 per cent in infected tomato plants. Exopolysaccharide from bacteria *Serratia* sp. strain Gsm01 showed antiviral activity against yellow strain of CMV in tobacco plants (Ipper *et al.,* 2006). Kavino *et al*. (2008) reported that application of *P. fluorescens* strain CHA0 along with chitin reduced the banana bunchy top disease incidence in banana under glasshouse and field conditions. Plant growth promoting microbial consortia treatment reduced sunflower necrosis virus disease (SNVD) incidence by lowering virus titre, apart from growth promotion (Srinivasan and Mathivanan, 2011). Patil *et al.* (2011) found that treatment of *P. fluorescens* strain 218 (1) to soil, seed and foliage reduced BYVMV incidence by 86.60 per cent in addition to increased growth and yield in okra.

2.6. ELUCIDATION OF ROLE OF OXIDATIVE AND SCAVENGING ENZYMES IN *P. indica*- PRIMED OKRA AGAINST BYVMV

Induced disease resistance is associated with the upregulation of antioxidant enzyme activities that scavenge the reactive oxygen species formed during pathogen infection; thus, protecting the cell. Enhanced glutathione reductase (GR) activity was observed in the *P. indica*–barley interaction; higher GR activity was associated with maintenance of antioxidant capacity by elevating levels of reduced glutathione (Waller *et al.,* 2005). Root colonization by *P. indica* elevated the amount of antioxidant enzymes viz., catalase (CAT), ascorbate peroxidase, dehydroascorbate reductase, monodehydro-ascorbate reductase and glutathione reductase (GST) in

barley roots under salt stress conditions (Baltruschat *et al*., 2008). Maize plants colonized with *P. indica* showed a 23, 3.8 and 1.7 - fold increase in activity of CAT, GST and super oxide dismutase (SOD) respectively compared to the non-colonized plants (Kumar *et al*., 2009). Sun *et al*. (2010) reported that *P. indica* colonized Chinese cabbage showed enhanced activities of peroxidase (PO), CAT, and superoxide dismutase (SOD) in the leaves within 24 h that led to increase in photosynthetic efficiency by preventing degradation of chlorophylls and thylakoid proteins under drought stress conditions. There was a 3.1fold increase in PAL activity in *P. indica*-colonized *Linum album* plants compared to the control plants which in turn increased lignan content (Kumar *et al.,* 2012a). AMF (*Funneliformis mosseae*) enhanced disease resistance against early blight in tomato by enhancing defense-related genes like *PAL, LOX, PR2, PR3, AOC, PR3* (Song *et al.,* 2015).

Ghorbani *et al.* (2018) reported that *P. indica*-colonised tomato plants showed improved activity of antioxidant enzymes like CAT, ascorbate peroxidase and GST activities under salinity stress. *P. indica* induced systemic resistance against rice sheath blight by decreasing the levels of hydrogen peroxide and by increased activity of superoxide dismutase (Nassimi and Taheri, 2017). *P. indica* primed cowpea plants showed increased activities of defense enzymes like peroxidase and polyphenol oxidase; thus, conferred protection against *Black eye cowpea mosaic virus* (Alex, 2017; Chandran, 2019). The activities of the antioxidant enzymes, CAT and GST were upregulated in *P. indica* inoculated rice seedlings under water stress (Tsai *et al*., 2020).

Banana bunchy top virus infected banana plants, treated with *P. fluorescens* CHA0 amended with chitin increased the accumulation of oxidative enzymes, peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), pathogenesis-related (PR) proteins along with increase in yield (Kavino *et al.,* 2008). Venkatesan *et al.* (2010) reported that black gram plants treated with *P. fluorescens* induced resistance against *Urd bean yellow mosaic virus* by enhancing the production of PO, PPO and PAL enzymes. *Pseudomonas chlororaphis* and *P. fluorescens* treated bittergourd plants showed a reduction in BYMV infection by enhanced production of PO, PPO and PAL enzymes (Rajinimala *et al.,* 2009). *P. fluorescens* was effective in

reducing tomato spotted wilt disease in tomato plants, by increased activity of polyphenol oxidase, b-1,3-glucanase and chitinase enzymes; a higher PPO activity was maintained up to 168 hrs after treatment (Kandan *et al.*, 2005).

The rhizobacteria, *P. fluorescens* reduced BYVMV disease incidence by triggering biosynthesis of defense enzymes like PO and PAL, whose activity were increased by 79 and 47 per cent respectively over diseased control (Patil *et al.*, 2011).

Materials and Methods

3. MATERIALS AND METHODS

The present research work entitled "Management of *Bhendi yellow vein mosaic virus* using beneficial fungal root endophyte *Piriformospora indica*" was carried out in the Department of Plant Pathology, College of Agriculture, Vellayani during the period 2018-2020, with the objectives of collection of BYVMV infected okra plants, its molecular detection, evaluation of the beneficial fungal root endophyte, *P. indica* for the management of BYVMV and to elucidate the role of antioxidants in the tripartite interaction. The materials and methods adopted for conducting the study are detailed in this chapter.

3.1 COLLECTION OF BYVMV INFECTED OKRA

Survey was conducted in three different locations of Thiruvananthapuram district *viz.*, Vellayani, Pappanchani and Palappur (Plate 1) for collection of BYVMV infected okra plants. The characteristic symptoms of disease developed were recorded in detail. Fifty plants were randomly selected from the fields and the disease scoring was done according to 0-5 scale developed by Bos (1982) with slight modification by Naveen, (2018) (Plate 5).

0 - No symptom

1 - Very mild mottling, initial vein clearing

2 - Mottling and complete yellowing of veins with green interveinal region

3 - Blisters and raised surface on leaves

4 - Distortion of leaves, curling with reduction in leaf size

5 - Stunting with negligible or no flowering and deformed pods

Plate 1. Different locations in Thiruvananthapuram district surveyed for the collection of BYVMV infected okra samples

0- no symptom

1 -Very mild mottling, initial vein clearing

2 - Mottling and complete yellowing of veins, interveinal regions green

3 - Blisters and raised surfaces on leaves

4 - Distortion of leaves, whole leaf become yellow and reduction in leaf size

5 -stunting with negligible or no flowering, yellowish and deformed fruits

Plate 5. Disease score chart of okra yellow vein mosaic disease (score 0-5) described by Bos (1982) with slight modification (Naveen, 2018)

The vulnerability index (V. I.) (Bos, 1982) and disease incidence (D. I.) in each location was calculated:

$$\text{Vulnerability Index} = \frac{(0n0+1n1+2n2+3n3+4n4+5n5) \times 100}{nt\,(nc-1)}$$

n0, n1……n5 - number of plants in the category of 0,1,2,3,4, and 5

nt - total number of plants

nc - number of categories

Per cent disease incidence was calculated as

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

The infected plant samples were collected and maintained in insect proof net house conditions.

3.2. SYMPTOMATOLOGY

The characteristic symptoms observed in BYVMV infected okra from each survey locations were studied and recorded.

3.3. MOLECULAR DETECTION OF BYVMV

3.3.1. Isolation of genomic DNA

The total DNA was also isolated using DNeasy plant mini kit (Qiagen, India; Cat. No. 69104) as per the manufacturer's protocol (www.qiagen.com/HB-1166). One hundred mg of fresh plant tissue was disrupted using liquid nitrogen in pre-chilled mortar and pestle and mixed with 400 µl of buffer AP1 and 4 µl RNase A. The samples were transferred to micro-centrifuge tubes, vortexed and incubated for 10 min at 65°C on a heating block. Tubes were inverted 2 - 3 times during incubation. 130 µl of buffer P3 was added to the samples, mixed well and incubated for 5 min on ice. Lysate was collected and centrifuged for 5 min at 14000 rpm. The supernatant was transferred into a QIAshredder spin column placed on a 2 ml collection tube and centrifuged for 2 min at 14,000 rpm. The flow-through was transferred into a new tube

without disturbing the pellet. Then added 1.5 volumes of buffer AW1 and mixed well by pipetting. 650 µl of the mixture was transferred into a DNeasy Mini spin column placed in a 2 ml collection tube. It was centrifuged for 1 min at 8000 rpm and the flow-through was discarded. This step was repeated with the remaining sample. The spin column was placed into a new 2 ml collection tube, added 500 µl of buffer AW2, and centrifuged for 1 min at 8000 rpm. Flow-through was discarded and another 500 µl of buffer AW2 was added. It was centrifuged for 2 min at 14000 rpm. The collection tube was carefully removed from the spin column so that the column does not come into contact with the flow-through. The spin column was transferred in to a new 2 ml micro-centrifuge tube and 100 µl of buffer AE was added into it for elution. This was kept for incubation for 5 min at room temperature (15-25°C) and centrifuged for 1 min at 8000 rpm. The last step was repeated and the isolated DNA was stored at -20 °C.

The quality and quantity of total DNA isolated was assessed using biospectrophotometer (Eppendorf, Germany). For this, 10 µl of extracted DNA sample was diluted in 1000 µl of distilled water and the absorbance was read at 260 and 280 nm. The quality of DNA obtained was calculated as ratio of O. D. 260 to O. D. 280 value, for high quality DNA sample the ratio of O. D. 260 to O. D. 280 value was found to be 1.8. Quantity of DNA was calculated as, 260 nm absorbance value x 100 ng μl^{-1}.

3.3.2. Agarose gel electrophoresis

Presence of DNA was confirmed by running the samples in 1.2 per cent agarose gel prepared in 1X TAE buffer with 2.7 µl of ethidium bromide and casted in a horizontal gel electrophoresis unit (Bio-Rad, USA). Eight µl of DNA was mixed with 2 µl of 6 X loading dye and 1 kb ladder (GeNei) and loaded into wells of the agarose gel. The gel was run at $75Vcm^{-1}$ in 1X TAE buffer (Appendix I) using the power pack unit (Bio-Rad, USA). After completion of run, the gel was visualized in a UV trans-illuminator system (Bio-Rad, USA) and documented in Gel Doc system (Gel DOC TM XR+, Bio-Rad, USA).

3.3.3 PCR amplification

The total DNA isolated was subjected to PCR amplification with the two universal primers, AV/AC and Deng (Table 4) specific to coat protein of BYVMV (*Begomovirus)*. The PCR amplification reactions were carried out in a 20 µl reaction mixture containing 10x PCR buffer (contains 1.5 mM $MgCl_2$) 2.0 µl, 10 mM dNTPs (dATP, dGTP, dCTP and dTTP) 1.0 µl, DNA 2.0 µl, Taq polymerase enzyme (2.5 Units) 0.5 µl, forward and reverse primers (5 pM) 1 µl each and 12.5 µl sterile distilled water. The reaction mixture was run in a thermocycler (Veriti 96 well Thermal cycler, Applied Biosystems, USA) under the specified conditions (Table 5).

3.3.4 Agarose gel electrophoresis of PCR products

The PCR products were run in 1.2 per cent agarose gels prepared in 1X TAE buffer containing 0.5 µg/ml ethidium bromide. Two µl of 6X loading dye was mixed with 8 µl of PCR products and loaded into the gel. The gel run was performed at $75Vcm^{-1}$ in 1X TAE buffer for 1 h. The molecular standard used was a 100 bp DNA ladder (GeNei, Bangalore, India). The gels were visualized and documented in gel documentation system.

3.4. CO-CULTIVATION OF OKRA WITH BENEFICIAL ROOT ENDOPHYTIC FUNGUS, *Piriformospora indica*

3.4.1. Maintenance of the fungal root endophyte *P. indica*

The beneficial fungal root endophyte, *P. indica* from Department of Plant Pathology, College of Agriculture, Vellayani was maintained in potato dextrose agar (PDA) medium (Appendix I). Fungal disc from actively growing margin of two weeks-old culture of *P. indica* was transferred to petri plates containing PDA and incubated in dark at room temperature. It was sub-cultured once in fifteen days. *In-vitro* and *in-vivo* co-cultivation of *P. indica* with okra var. Salkeerthi were carried out as per the procedure described by Johnson *et al.* (2013).

Table 4. PCR conditions used for the amplification of coat protein gene of BYVMV with Deng and AV/AC primers

Stage	Function	Temperature (^{o}C)	Time (minute)	No. of cycles
I	Initial denaturation	94	3	1
II	Denaturation Primer Annealing Primer extension	94 58 72	1 1 1	35
III	Final extension	72	10	1

Table 5. Universal primers used for amplification of coat protein gene specific for *Geminivirus*

Primer	Sequences (5'-3')	Product (bp)	References
AV 494-F AC 1048-R	GCCHATRTAYAGRAA GCCMAGRAT GGRTTDGARGCATGHGTACANG CC	575	Wyatt and Brown (1996)
Deng 541-F Deng 540-R	TAATATTACCKGWKGVCCSC TGGACYTTRCAWGGBCCTTCACA	520	Deng *et al*. (1994)

3.4.2. *In-vitro* co-cultivation of *P. indica* with okra

Modified PNM medium (Appendix II) was prepared, autoclaved and filled 40 ml in sterilized jam bottles; and allowed to solidify. Five mm mycelial discs from two week- old culture of *P. indica* was placed in the centre of jam bottle containing solidified media and incubated in dark. Okra seeds were surface sterilized in 0.1 per cent mercuric chloride for 7 min, followed by 3 washings in sterile water and dried. Four surface sterilized seeds were transferred to each jam bottles containing modified PNM media with sufficient fungal growth and kept for incubation at 24°C.

3.4.3. *In-vivo* co-cultivation of *P. indica* with okra in vermiculite-perlite medium

Potting mixture containing vermiculite and perlite in 3:1 ratio was used for *in-vivo* co-cultivation studies. The medium was moistened up to field capacity and sterilized at 121°C for 20 min for two consecutive days. *P. indica* was cultured in 100 ml potato dextrose broth in 250 ml conical flasks and incubated at room temperature with constant shaking in orbital shaker at 40 rpm for 15 days. Fungal mycelium was harvested by filtration through double layered muslin cloth and washed with two changes of sterile water. Mycelial mass was mixed with sterile potting media at 1 per cent (w/v), filled in trays, covered and kept for fungal growth. After one week, surface sterilized okra seeds were placed in portrays filled with medium and maintained in temperature and humidity - controlled conditions for uniform germination.

3.4.4. Root colonization of okra roots var. Salkeerthi by *P. indica*

Roots of okra were collected at 10, 15, 20 and 25 days after co-cultivation. Roots were washed thoroughly with running water to make it free from planting medium. Roots were cut into small pieces of 1 cm length and transferred into test tube containing 5 ml of freshly prepared 10 per cent KOH. It was placed in water bath at 65°C for 5 min. Roots were washed once with water and treated with 1 per cent HCl for 5 min. Root bits were again washed with water and transferred into lactophenol trypan blue for 2 min to stain the fungus in roots. The root bits were observed under microscope (Leica - ICC50 HD, USA) for the presence of chlamydospores and colonization in each root bit.

3.5. EVALUATION OF *P. indica*-PRIMED OKRA SEEDLINGS AND PLANTS AGAINST BYVMV

3.5.1. Evaluation of *P. indica*-primed okra seedlings and plants under natural incidence of BYVMV

A pot culture experiment was conducted for evaluating *P. indica*- primed okra var. Salkeerthi against natural incidence of BYVMV (Plate 12) The experiment consisted of two treatments, and 10 plants in each treatment. For this *P. indica* primed and non-primed plants were raised following procedure described earlier in 3.4.3. BYVMV infected plants were maintained around the experimental plot that served as inoculum. Observations were taken for symptom development, disease incidence, vulnerability index and biometric characters.

3.5.2. Evaluation of *P. indica*-primed okra seedlings and plants against artificial inoculation of BYVMV by grafting

A pot culture experiment was laid out in completely randomized design (CRD) with five treatments and five replications per treatment, for evaluating *P. indica* against BYVMV in okra var. Salkeerthi.

T1: Absolute control

T2: *P. indica*-primed okra seedling alone

T3: Graft transmission of the virus alone

T4: *P. indica*-priming followed by graft transmission of the virus

T5: Graft transmission of the virus followed by *P. indica*-priming

For pre-inoculation studies, sterilized potting mixture was taken and mixed with 1 per cent *P. indica* mycelial mass, filled in trays, covered and kept for fungal growth. After one week, surface sterilized okra seeds were placed in portrays filled with the medium and maintained in temperature and humidity - controlled conditions for uniform germination. One week-old seedlings were transferred to pots. Graft transmission BYVMV was done after 25 days of sowing by wedge grafting method. For this, infected twigs were taken as scion and 5 - 6 leaved healthy plants were used as rootstock for grafting. The apical portion of rootstock was cut horizontally and

Plate 12. General view of pot culture experiment for evaluation of *P. indica*-primed okra plants var. Salkeerthi against natural incidence of BYVMV

removed in such a way that minimum two nodes remain intact to the rootstock. The cut end of scion was gently made into a wedge shape and inserted into the gentle vertical incision made in the stock. The graft union was then made tight using polythene strip or grafting tape and graft was covered with polythene cover for 3 - 4 days. Grafted seedlings were kept inside insect proof cages.

For post-inoculation of *P. indica*, okra seedlings were raised in portrays filled with sterilized potting mixture containing vermiculite and perlite in the ratio 3:1 and transferred to pots one week after sowing. After 25 days, graft transmission of BYVMV was done using wedge grafting method. *P. indica* multiplied in potting mixture was applied in the root zone after grafting. Observations were taken for symptom development, disease incidence and vulnerability index.

3.6. ELUCIDATION OF ROLE OF OXIDATIVE AND SCAVENGING ENZYMES IN *P. indica*- PRIMED OKRA AGAINST BYVMV

Biochemical changes were analysed separately for natural incidence, post- and pre- inoculation experiment by collecting samples at 40 days after sowing from naturally infected plants and 30 days after grafting from artificially inoculated plants. The biochemical studies included analysis of total chlorophyll, total soluble protein, peroxidase, polyphenol oxidase, catalase, ascorbic acid oxidase and phosphatase.

3.6.1. Total chlorophyll content

Total chlorophyll content was estimated following the method described by Arnon (1949). One gram of leaf sample was ground in 20 ml of 80 per cent acetone and centrifuged at 5000 rpm for 5 min. The supernatant was transferred to 100 ml volumetric flask. The residue was again ground in 80 per cent acetone, centrifuged and supernatant was transferred to volumetric flask. The process was repeated till the residue become colourless. The volume of volumetric flask was made up to 100 ml with 80 per cent acetone. Absorbance of solution was measured at 645 and 663 nm against solvent blank (80 per cent acetone).

Total chlorophyll, chlorophyll a and chlorophyll b contents were calculated according to the following equations:

$$\text{Chlorophyll a (mg/g)} = \frac{12.7\,(A663) - 2.69\,(A645)}{1000 \times W} \times V$$

$$\text{Chlorophyll b (mg/g)} = \frac{22.9\,(A645) - 4.68\,(A663)}{1000 \times W} \times V$$

$$\text{Total chlorophyll (mg/g)} = \frac{20.2\,(A645) + 8.02\,(A663)}{1000 \times W} \times V$$

V: final volume of chlorophyll extract

W: fresh weight of the leaves

3.6.2. Total soluble protein

Total soluble protein was estimated by protocol described by Bradford (1976). One gram of leaf sample was homogenised in 10 ml of 0.01 M sodium acetate buffer (pH 4.7) (Appendix III), centrifuged at 5000 rpm for 15 min at 4ºC. Supernatant was collected for protein estimation. Reaction mixture consisted of 0.5 ml enzyme extract, 0.5 ml distilled water and 5 ml of dye solution (Appendix III). Blank was prepared by adding 1 ml of distilled water with 5 ml of dye solution. The absorbance was measured at 595 nm in a spectrophotometer against the reagent blank. Standard solution was prepared by using bovine serum albumin. The protein content present in the sample was expressed in microgram albumin equivalent of soluble protein per gram on fresh weight basis.

3.6.3. Peroxidase

Peroxidase (PO) activity was determined by a protocol described by Srivastava (1987). One gram of leaf was homogenised with 5 ml of sodium phosphate buffer (pH 6.5) (Appendix IV) with a pinch of poly vinyl pyrrolidone (PVP) in chilled pestle and mortar. It was filtered with cheese cloth and centrifuged at 6000 rpm for 15 min at 4°C. The supernatant was collected for analysis of PO activity. The reaction mixture consists of 3 ml of 0.05 M pyrogallol, 50 µl of enzyme extract in sample cuvette and pyrogallol in reference cuvette. The reaction was initiated by adding 1 ml of one per

cent hydrogen peroxide into sample cuvettes and changes in absorbance was measured in spectrophotometer at 420 nm in 30 seconds interval for 180 seconds.

3.6.4. Polyphenol oxidase

Poly phenol oxidase (PPO) activity was assessed by following the procedure described by Mayer *et al.* (1954). One gram of leaf was ground in 5 ml of sodium phosphate buffer (pH 6.5) (Appendix IV) with poly vinyl pyrrolidone in pre-chilled pestle and mortar in 4°C. It was filtered through cheese cloth and centrifuged at 6000 rpm for 15 min at 4°C. The supernatant was used for the assay of PPO activity. The reaction mixture contained 1ml of sodium phosphate buffer, 200 µl enzyme extract in sample cuvette and buffer alone for reference cuvette, kept in spectrophotometer. The reaction was initiated by adding 1 ml of 0.01 M catechol. Change in absorbance was measured in spectrophotometer at 495 nm with 30 seconds interval for 180 seconds.

3.6.5. Catalase

Catalase activity was determined by procedure described by Luck (1974). 1 g of leaf tissue was homogenised in 20 ml of 0.0067 M phosphate buffer (pH 7.0) (Appendix IV). It was centrifuged at 5000 rpm for 15 min at 4ºC. The extraction was repeated twice. The supernatants were combined and used for the enzyme assay. 40 µl of the extract was added to the sample cuvette containing 3 ml H_2O_2 - PO_4 buffer while H_2O_2 - free PO_4 buffer in control cuvette (Appendix V) and the time taken for the change in absorbance (Δt) by 0.05 at 240 nm was recorded for calculating the enzyme units per ml of the extract.

3.6.6. Ascorbic acid oxidase

Ascorbic acid oxidase was determined by protocol developed by Oberbacher and Vines, (1953). One gram of leaf tissue was macerated in 5ml 0.1 M phosphate buffer (pH 6.5) (Appendix IV) in prechilled pestle and mortar. The homogenate was centrifuged at 5000 rpm for 15 min. The supernatant was collected and used for enzyme assay. The reaction mixture consists of 0.1 ml enzyme extract, 3 ml substrate (ascorbic acid) solution in sample cuvette and 3ml of substrate solution in reference cuvette. The absorbance was read at 265 nm in 30 seconds interval for 5 min.

3.6.7. Phosphatase

Phosphatase activity was determined by the method described by Lowry *et al.* (1954). One g of fresh plant tissue was taken and homogenized in 10 ml of ice-cold 50 mM citrate buffer (pH 5.3) (Appendix IV) in a pre-chilled pestle and mortar; and filtered through 4 layered cheese cloth. The filtrate was centrifuged at 12000 rpm for 10 min and the supernatant was used for enzyme assay. Three ml substrate solution (pH 5.3) was incubated at 37^{o}C for 5 min; and to this 0.5 ml enzyme extract was added and mixed well. From this solution, 0.5 ml was removed immediately and mixed with 9.5 ml of 0.085 N sodium hydroxide which corresponds to blank. The remaining solution was incubated for 15 min at 37^{o}C and 0.5 ml sample was taken from this and mixed with 9.5 ml of sodium hydroxide. Absorbance of this solution was measured at 405 nm against blank. Standard solution was prepared by using *p*-nitrophenol. Specific activity of phosphatase was expressed as m moles of *p*-nitrophenol produced per min per g fresh weight of tissue.

All the experiments were statistically analysed to draw meaningful conclusions.

Results

4. RESULTS

The current research work entitled “Management of *Bhendi yellow vein mosaic virus* using beneficial fungal root endophyte *Piriformospora indica*” was carried out in the Department of Plant Pathology, College of Agriculture, Vellayani during the period 2018 - 2020, to evaluate the beneficial fungal root endophyte, *P. indica* for the management of BYVMV and to elucidate the role of antioxidants in the tripartite interaction. The results of the study are detailed in this chapter.

4.1 COLLECTION OF BYVMV INFECTED OKRA

A survey was conducted in three different locations of Thiruvananthapuram district *viz.*, Vellayani, Pappanchani and Palappur during July 2018 – March 2020, to collect BYVMV infected okra samples and to assess the disease incidence and severity of BYVMD (Plate 1). Various symptoms observed, disease incidence and vulnerability index (V. I.) in each location were recorded. V. I. for BYVMD was calculated using score chart mentioned in Plate 2. Samples were collected randomly from each field for the further symptomatological studies and molecular detection.

All the surveyed locations were severely infected with BYVMV. The BYVMD incidence in the various surveyed locations varied from 67 to 100 per cent (Table 2). The highest disease incidence of cent per cent was recorded from Palappur for the okra variety Kiran with maximum V. I. value of 71.07 (Table 2). This was followed by Pappachani where a D. I. of 91 per cent was recorded for okra variety Aruna with V. I. of 68.4 (Table 2). Among the surveyed locations lowest D. I. and V. I. of 67 per cent and 32.60 respectively were recorded from Vellayani for the okra variety Anjitha (Table 2).

Table 1. Symptoms of BYVMD observed in different survey locations

Sl. No.	Location	Symptoms observed
1	Vellayani	Mottling and complete yellowing of veins; and bleached and malformed fruits
2	Pappanchani	Mottling, vein clearing, vein thickening; stunted growth and reduced fruit set; and bleaching and hardening of fruits
3	Palappur	Vein clearing, vein thickening, distorted and small sized leaves with slight curling; vein clearing on flower buds and bleached appearance of fruits

Table 2. Disease incidence and vulnerability index of BYVMV isolates collected from different locations

Sl. No	Location	Isolate name	Variety	D.I (%)	V.I
1	Vellayani	BYVMV Vellayani	Anjitha	67	32.60
2	Pappanchani	BYVMV Pappanchani	Aruna	91	68.40
3	Palappur	BYVMV Palappur	Aruna	100	71.07

D. I. - Disease incidence; V. I. - Vulnerability index

4.2 SYMPTOMATOLOGY

Characteristic symptoms of BYVMD developed in different okra varieties in survey locations were studied as shown in Table 1. Mottling and vein clearing of leaves, reduction of leaf size, stunted growth, and bleached, hardened and malformed fruits were observed in all surveyed locations (Plate 2, Plate 3 and Plate 4). In addition to these symptoms, vein thickening distorted leaves with slight curling was observed in Pappanchani and Palappur. Apart from this, vein clearing on flower buds was observed in Palappur (Plate 4).

4.3. MOLECULAR DETECTION OF BYVMV

The total DNA from infected plant samples were isolated using DNeasy plant mini kit (QIAGEN: Cat. No. 69104). Presence of DNA was confirmed by running the samples in 1.2 per cent agarose gel and visualised in UV trans-illuminator system (Bio-Rad). The quantity and quality of total DNA isolated was assessed in bio-spectrophotometer. Among the DNA samples, BYVMD from Vellayani recorded the quality of 1.31 with quantity of 564.3 ng μl^{-1}. Least quality was recorded for BYVMD of Palappur isolate with DNA quantity of 613.8 ng μl^{-1} (Table 3).

The isolated DNA was subjected to PCR using two universal primers (AV/AC and Deng) (Table 5) specific to coat protein of BYVMV (*Begomovirus*) under specified conditions (Table 4). All the isolates yielded amplicon size of 575 bp with AV/AC primer (Plate 4) and 520 bp with Deng primer (Plate 5, Table 6). The DNA isolated from pollen grains of BYVMV infected plant could also produce amplicons of 575 and 520 bp with AV/AC and Deng primer respectively, thus confirmed the presence of virus (Plate 6 and 7).

Table 3. Quality and quantity of DNA of BYVMV isolates

Sl. No.	Isolate	OD at 260 nm	OD at 280 nm	260/280 value	Quantity of DNA (ng μl^{-1})
1	BYVMV Vellayani	0.171	0.130	1.31	564.3
2	BYVMV Pappanchani	0.110	0.084	1.30	363
3	BYVMV Palappur	0.186	0.153	1.21	613.8

OD- Optical Density

Table 6. Molecular detection of BYVMV isolates using AV/AC and Deng primers

Sl. No.	Isolate	Primer	
		AV/AC	Deng
1	BYVMV Vellayani	+	+
2	BYVMV Pappanchani	+	+
3	BYVMV Palappur	+	+

Field 1

Vein clearing

Bleached fruits

Field 2

Vein clearing

Reduction in leaf size, bleached and deformed fruits

Plate 2. Symptoms of BYVMD observed in different fields of Vellayani, Thiruvananthapuram

Field 3

Mild vein clearing

Distortion and reduction in leaf size

Plate 3. Symptoms of BYVMD observed in Pappanchani, Thiruvananthapuram

Field 4

Vein clearing on flower buds

Reduction in leaf size, bleached and deformed fruits

Plate 4. Symptoms of BYVMD observed in Palappur, Thiruvananthapuram

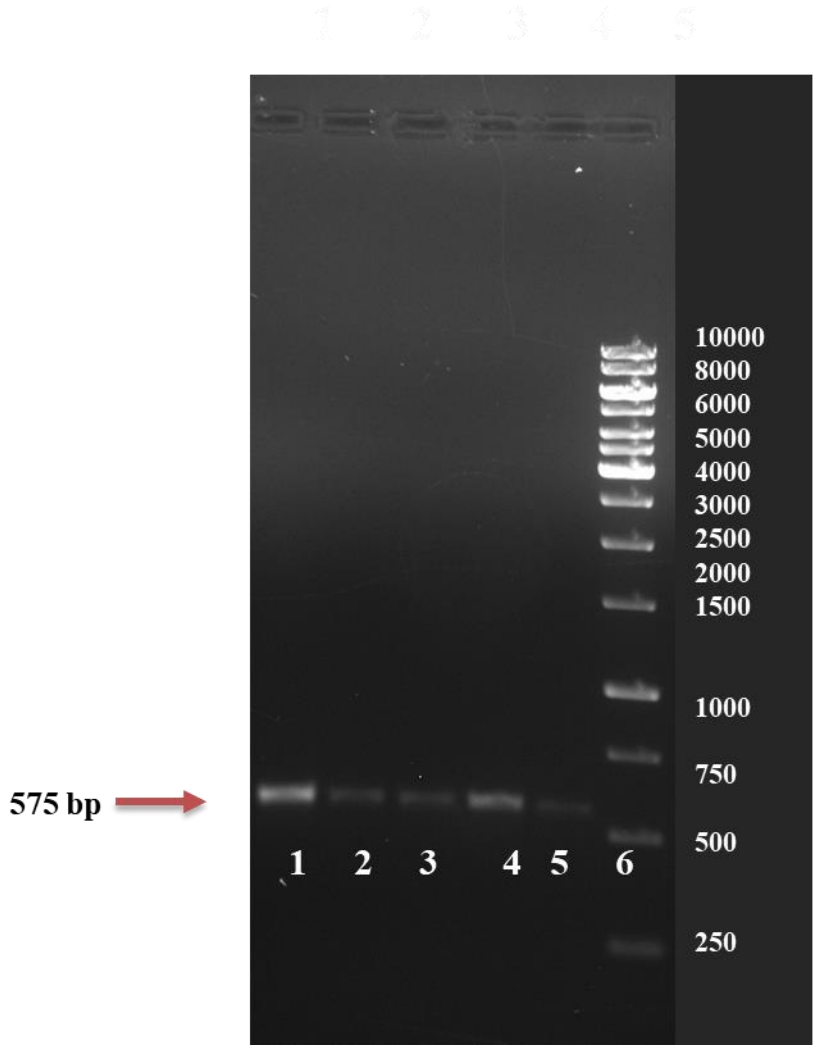


Plate 6. Electrophoresis gel image of amplified DNA of BYVMV using AV/AC primer. Lane 1) & 2) BYVMV Vellayani, 3) BYVMV Pappanchani, 4) BYVMV Palappur, 5) Pollen grain sample of infected plants, 6) 1 kb marker

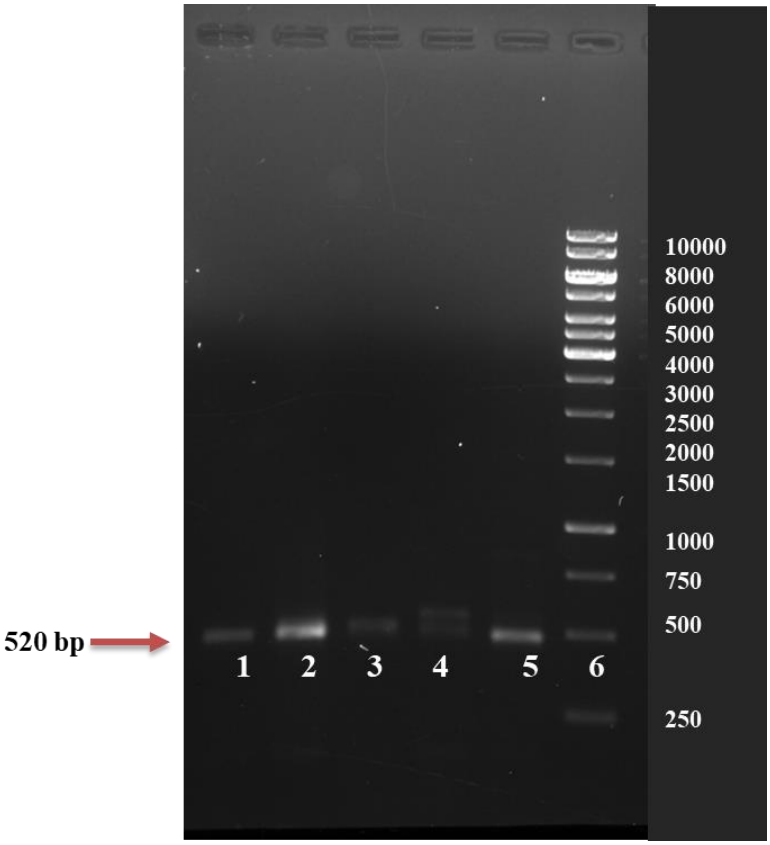


Plate 7: Electrophoresis gel image of amplified DNA of BYVMV using Deng primer. Lane 1) & 2) BYVMV Vellayani, 3) BYVMV Pappanchani, 4) BYVMV Palappur, 5) Pollen grain sample of infected plants, 6) 1 kb marker

4.4. CO-CULTIVATION OF OKRA WITH BENEFICIAL ROOT ENDOPHYTIC FUNGUS, *Piriformospora indica*

4.4.1. Maintenance of the fungal root endophyte *P. indica*

P. indica was maintained in potato dextrose agar (PDA) medium by regular sub-culturing once in 3-4 weeks. Fungus attained full growth in petri-plate after 10 days of inoculation (Plate 5). Fungal growth in potato dextrose broth after 18 days of inoculation was depicted in Plate 5.

4.4.2. *In vitro* co-cultivation of *P. indica* with okra

In-vitro co-cultivation of *P. indica* with okra was done in modified PNM medium. Surface sterilized seeds were placed in jam bottles containing PNM medium previously inoculated with *P. indica.* Microscopic examination of roots at various intervals revealed presence of the mycelium in root surface after 10 days of co-cultivation and chlamydospores were produced on root surface after 15 days of co-cultivation (Plate 9). Colonisation inside roots was observed after 20 days of co-cultivation. Extensive colonisation with mature chlamydospores in chains was observed within the root cells after 25 days of co- cultivation (Plate 9).

Growth promotion of okra seedlings var. Salkeerthi colonised by *P. indica* was assessed at different intervals of the co-cultivation. *P. indica* colonisation resulted in increase in root and shoot biomass, and production of more number of secondary roots. Shoot and root biomass was significantly higher in colonised plants after 10, 15, and 20 days of co-cultivation, marked difference being found after 20 days of co-cultivation. Colonised plants showed 29 and 50 per cent increase in the shoot and root biomass respectively over the non-colonised plants after 20 days of co-cultivation (Table 7). Root length and number of secondary roots were also increased due to colonisation. Longer and robust roots were recorded in *P. indica*-colonised plants (average root length of 12.6 cm) while it was shorter in non-colonised plants (8.48 cm) after 20 days of co-cultivation.

Table 7. Shoot and root biomass of okra seedlings var. Salkeerthi colonised by *P. indica*

Treatments	Shoot biomass (g)				Root biomass (g)			
	5 DAC	10 DAC	15 DAC	20 DAC	5 DAC	10 DAC	15 DAC	20 DAC
- *P. indica*	0.28	0.34	0.37	0.41	0.03	0.07	0.08	0.10
+ *P. indica*	0.29	0.39	0.49	0.53	0.04	0.09	0.13	0.15
T- calculated	2.29	4.45	5.53	11.80	2.66	9.14	16.78	10.86
T (0.05)	2.306							

DAC- Days after co-cultivation; Values are mean of 5 observations

Table 8. Root length and number of secondary roots in okra seedlings var. Salkeerthi colonised by *P. indica*

Treatments	Root length (cm)				Number of secondary roots			
	5 DAC	10 DAC	15 DAC	20 DAC	5 DAC	10 DAC	15 DAC	20 DAC
- *P. indica*	5.36	6.14	7.52	8.48	8.4	22.2	28.6	32.2
+ *P. indica*	5.88	7.02	9.00	10.45	12.6	32.4	42.8	47.6
T – calculated	1.78	2.6	5.53	7.78	3.80	5.23	6.16	6.75
T (0.05)	2.306							

DAC- Days after co-cultivation; Values are mean of 5 observation

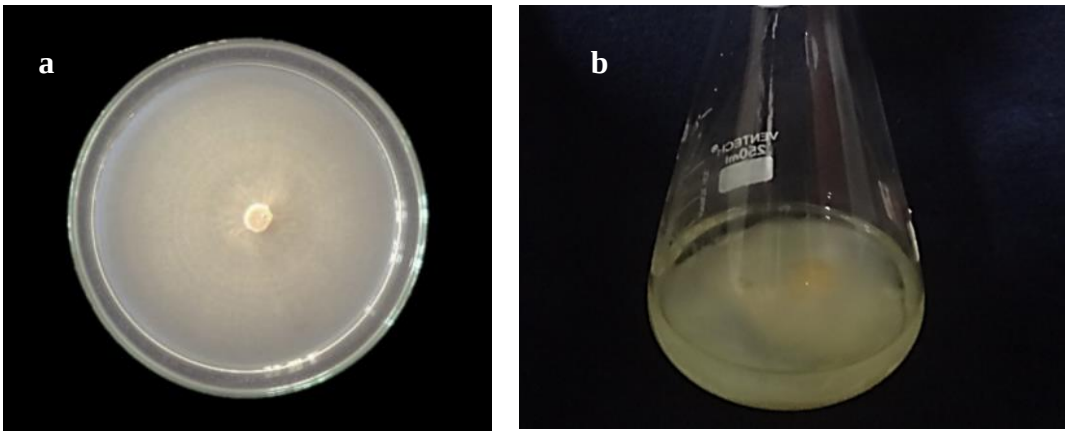
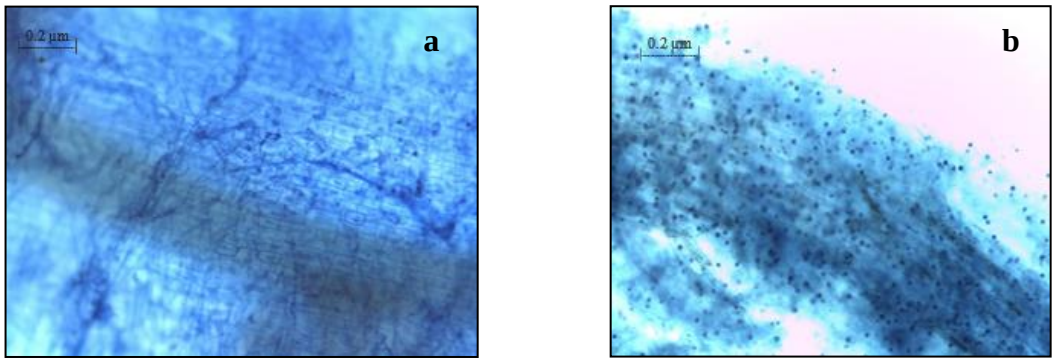
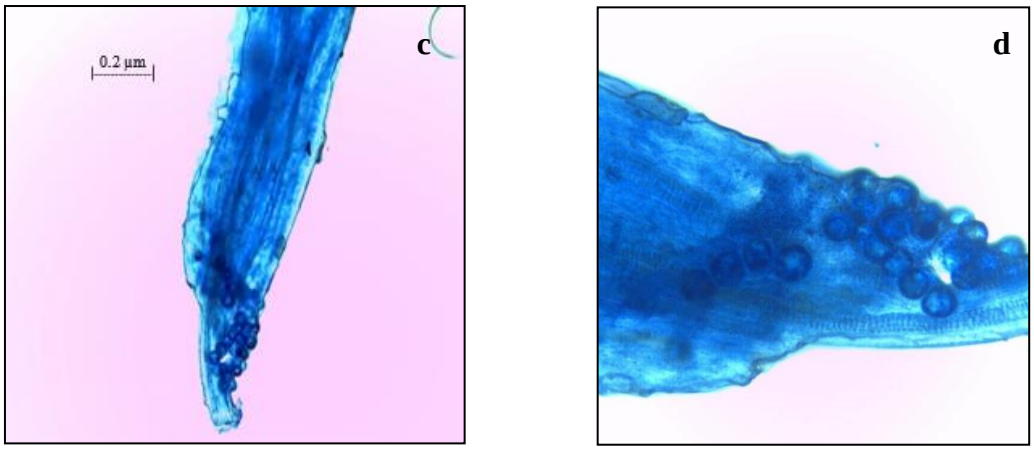


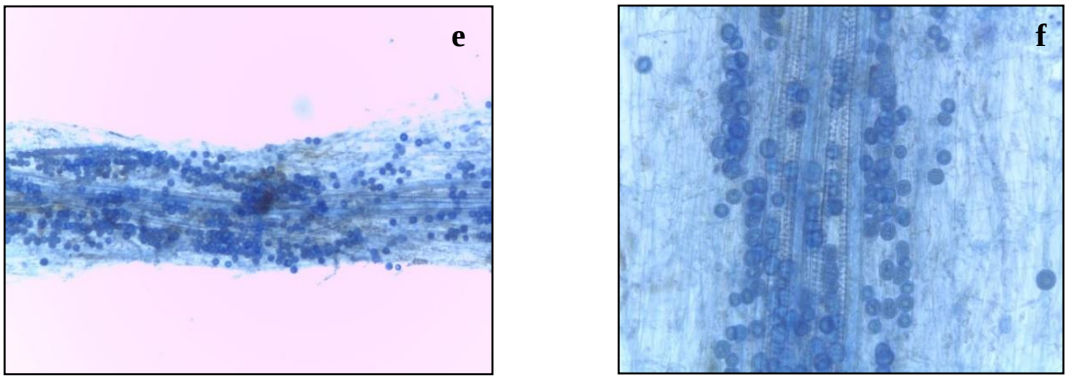
Plate 8. Maintenance of *P. indica* in potato dextrose agar (PDA) medium
(a) Growth of *P. indica* in potato dextrose agar - 10 days after inoculation;
(b) Growth of *P. indica* in potato dextrose broth - 18 days after inoculation



100 X 100 X



100 X 400 X



100 X 400 X

Plate 9. *In vitro* root colonization of *P. indica* in okra in PNM medium. (a) Mycelium on root surface at 10 DAC (Days after co-cultivation); (b) Chlamydospores on root at 15 DAC; (c) and (d) *P. indica* colonisation in root at 20 DAC; (e) and (f) *P. indica* colonisation in root at 25 DAC

Colonisation of *P. indica* resulted in 49 per cent increase in secondary roots formation compared to the control plants (Table 8). Thus *in vitro* co- cultivation of okra seedlings with *P. indica* in PNM medium revealed its colonisation in roots; and formation of chlamydospores after 25 days. *P. indica* colonisation enhanced growth parameters such as shoot and root biomass, and increased the number of secondary roots in okra seedlings.

4.4.3. *In vivo* co- cultivation of *P. indica* with okra in vermiculite-perlite medium

In vivo co-cultivation of *P. indica* with okra var. Salkeerthi was done in potting mixture mixed with 1 per cent mycelium of P. *indica* in portrays and maintained under temperature- and humidity- controlled conditions for uniform germination. Roots were assessed for *P. indica* colonisation at different intervals. Chlamydospores were seen inside the root cells after 25 days of co-cultivation (Plate 10). *P. indica* colonisation also enhanced growth of okra seedlings under *in vivo* conditions (Plate 11).

4.5. EVALUATION OF *P. indica*- PRIMED OKRA SEEDLINGS AND PLANTS AGAINST BYVMV

4.5.1. Evaluation of *P. indica*-primed okra seedlings and plants under natural incidence of BYVMV

A pot culture experiment was conducted for evaluating the effect of *P. indica*-primed okra plants var. Salkeerthi under natural incidence of BYVMV (Plate 9). *P. indica* root colonisation in okra plants could significantly reduce the BYVMD incidence (30 per cent) and severity (24.0) (Plate 13, Plate 14); whereas in non-colonized plants it was 70 per cent and 54 respectively after 40 days of sowing (Table 9). Thus, *P. indica*- priming reduced the D. I. by 57 per cent and V. I. by 56 per cent over the control plants after 40 days of sowing. The reduction in D. I. and V. I. was 40 per cent and 53 per cent respectively after 55 days of sowing; indicating the effect of *P. indica* priming was more prominent in early stage of crop (Table 12).

Table 9. Effect of *P. indica*-priming on natural incidence and severity of BYVMD in okra var. Salkeerthi

Treatments	Disease incidence (%)		Vulnerability index	
	40 DAS	55 DAS	40 DAS	55 DAS
-*P. indica* + BYVMV	70	100	54	78
+ *P. indica* + BYVMV	30	60	24	36

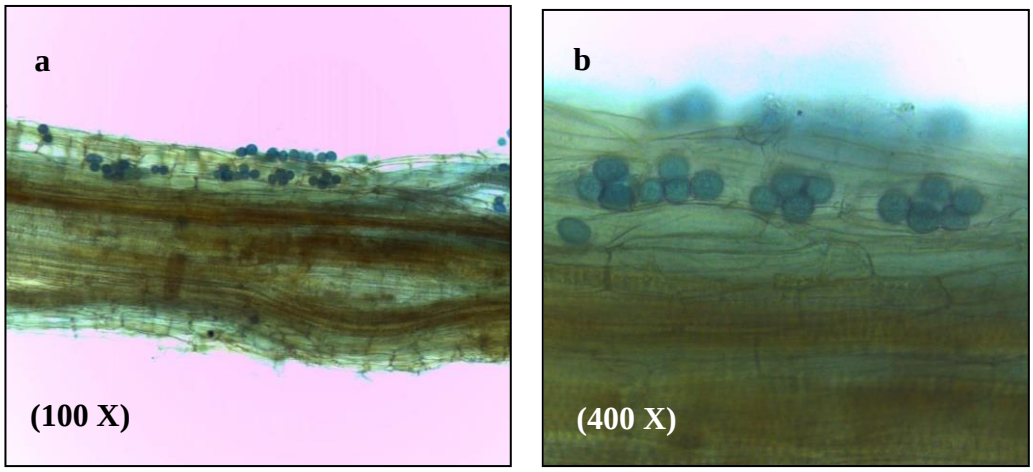
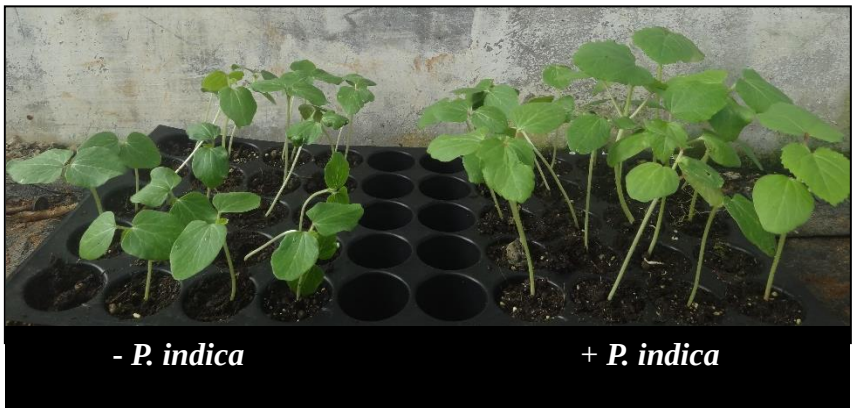


Plate 10. a) and b) - *In vivo* root colonization of *P. indica* in okra 25 DAC



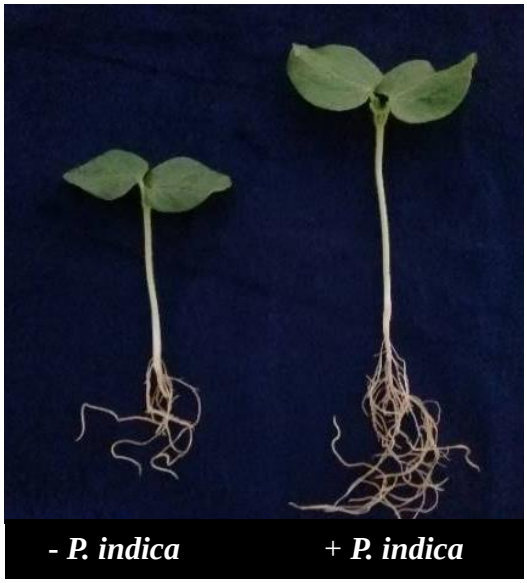


Plate 11. Growth promotion of okra var. Salkeerthi by *P. indica* under *in-vivo* conditions at seven day after sowing.

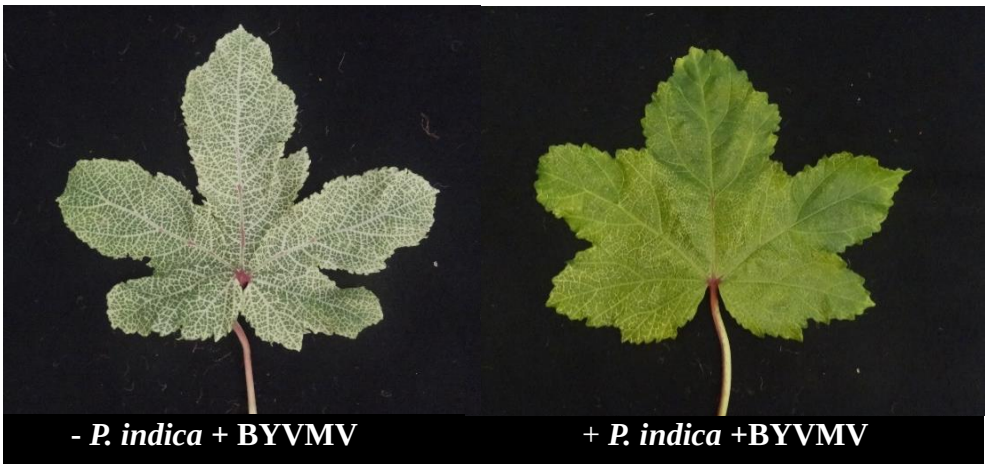


Plate 13. Severity of BYVMD in *P. indica* non-primed and -primed okra against natural incidence of BYVMV at 40 DAS

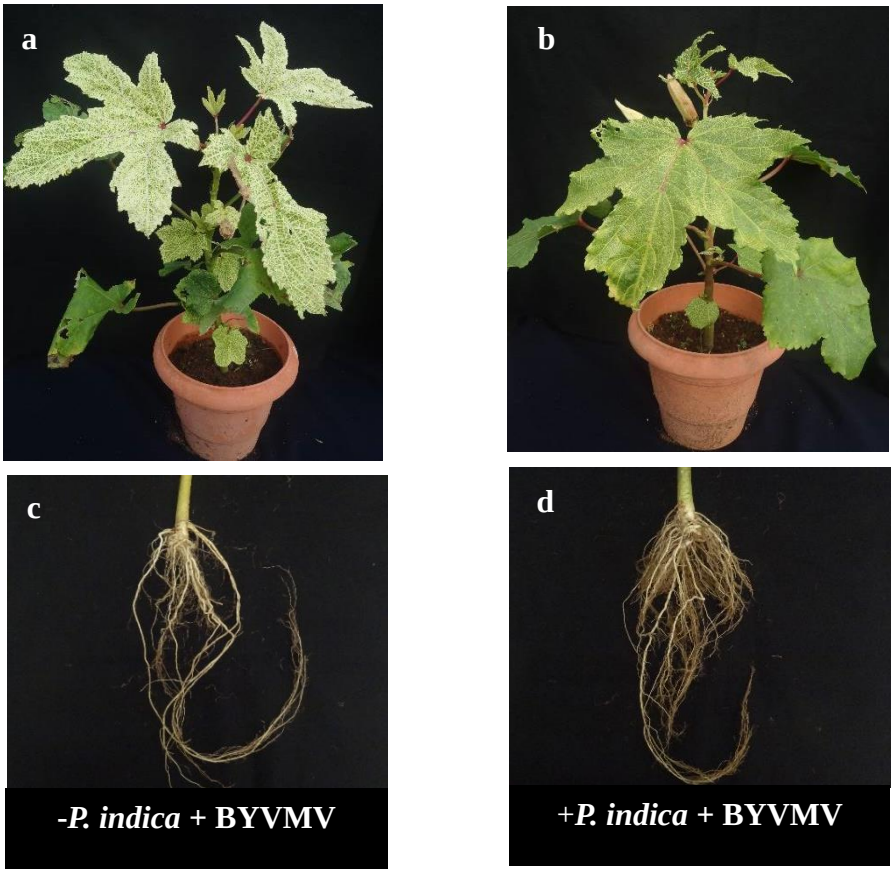


Plate 14. Effect of *P. indica*-priming on growth of okra plants var. salkeerthi under natural incidence of BYVMV at 40 DAS. a) BYVMD in *P. indica* non-primed okra plant; b) *P. indica*-primed plant; c) and d) Root growth in *P. indica*-non primed and primed okra plant

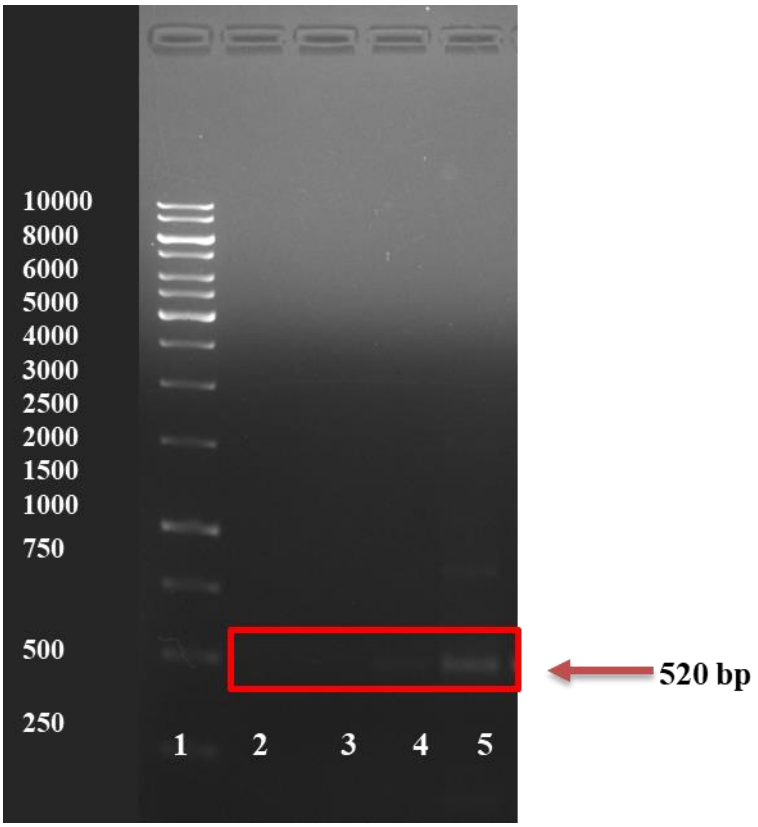


Plate 15. Electrophoresis gel image of amplified DNA of BYVMV using Deng primer; Lane 1) 1 kb marker, 2) Healthy (control), 3) + *P. indica* alone 4) + *P. indica* + BYVMV (mild symptom) 5) - *P. indica* + BYVMV (severe symptom)

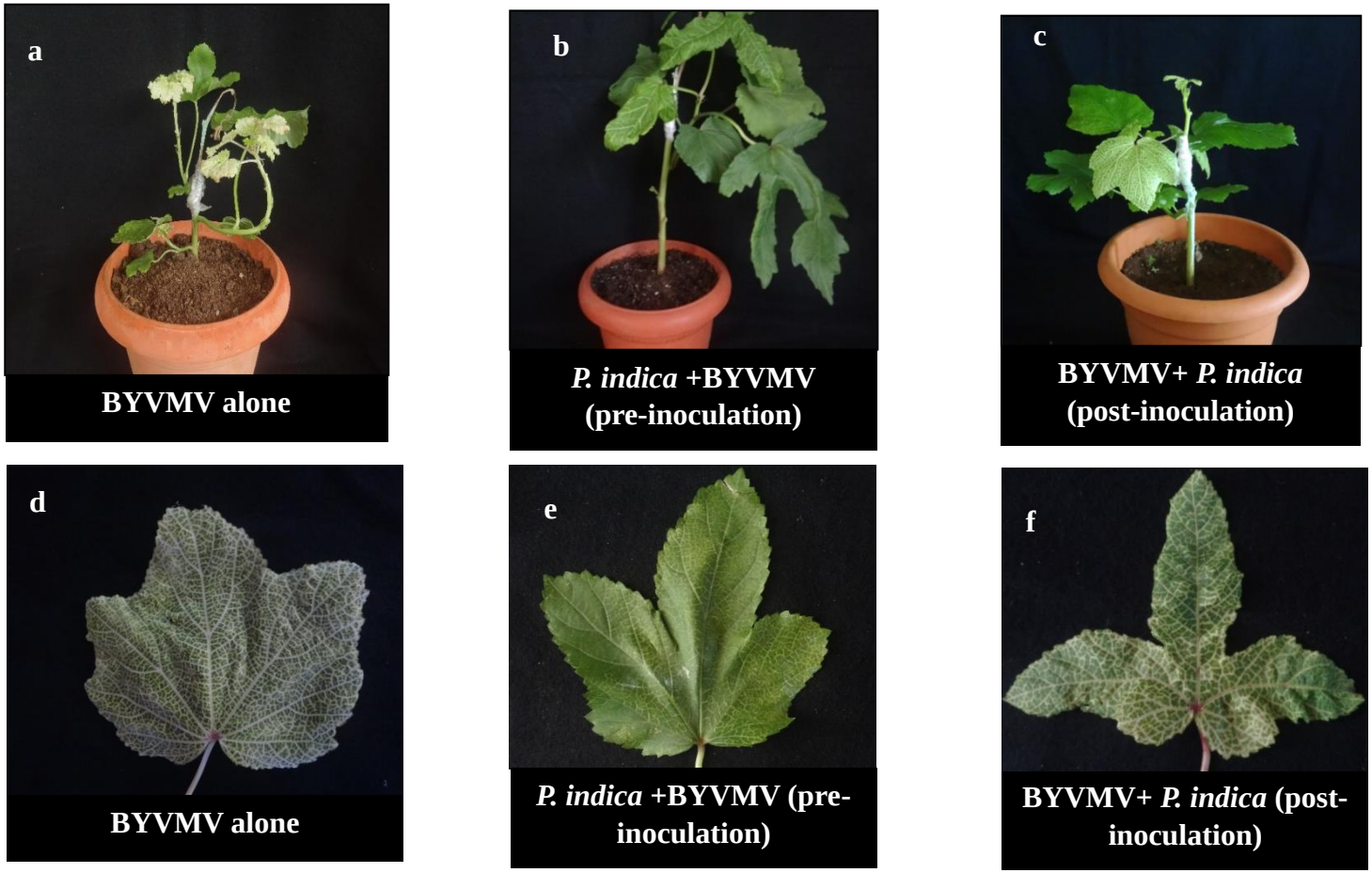


Plate 16. Effect of *P. indica*-priming in okra against BYVMV on graft transmission at 30 DAG (Days after grafting): a) and d) graft transmission alone b) and c) *P. indica* priming followed by graft transmission c) and f) graft transmission followed by *P. indica* priming

Biometric parameters like plant height, leaf area, number of leaves, and shoot and root biomass were assessed after 40 days of sowing under natural incidence of BYVMV (Table 10). All parameters were significantly higher in *P. indica*-primed plants than in control plants except number of leaves. Average plant height was significantly higher in *P. indica*-colonised plants (43.72 cm) compared to the non-colonised plants (36.28 cm). *P. indica*-primed plants exhibited larger leaf area (208.09 cm^2) compared to the non-primed plants (167.14 cm^2). There was 48 per cent and 62 per cent increase in root and shoot fresh weight respectively in *P. indica*-colonised plants over the control. Accordingly, root and shoot dry weight was also higher in *P. indica*-colonised plants (Table 10).

PCR analysis of infected okra plants revealed that viral accumulation was less in *P. indica* colonised plants compared to non- colonised diseased (Plate 15).

4.5.2. Evaluation of *P. indica*- primed okra seedlings and plants against artificial inoculation of BYVMV by grafting

A pot culture experiment was laid out in completely randomized design (CRD) with five treatments and five replications per treatment, for evaluating *P. indica*-primed okra var. Salkeerthi against artificial inoculation BYVMV by grafting. For pre-inoculation, okra plants primed with *P. indica* were grafted after 25 of sowing by wedge grafting. For post-inoculation fungus multiplied in potting mixture was applied in root zone following the grafting.

Days taken for symptom appearance and vulnerability index were assessed after 30 days of grafting. *P. indica*-priming significantly reduced the severity of symptoms of BYVMV under artificial inoculation by grafting (Plate 16). Least V. I. was shown by pre-inoculated plants (32.50), followed by post-inoculated plants (56.00) and the highest V. I. by *P. indica* non-primed diseased plants (74.00) (Table 14). Thus, pre- and post- inoculation of the fungus in artificially infected okra plants reduced the V. I. by 56 per cent and 24 per cent respectively over the diseased control.

Table 10. Effect of *P. indica*-priming on growth parameters of okra plants under natural incidence of BYVMV at 40 DAS

Treatments	Plant height (cm)	Leaf area (cm^2)	Number of leaves	Shoot biomass (g)		Root biomass (g)	
				FW	DW	FW	DW
-*P. indica* + BYVMV	36.28	167.14	7.60	87.96	12.83	9.18	1.46
+ *P. indica* +BYVMV	43.72	208.09	8.40	129.70	18.83	12.98	2.08
T – calculated	6.029	4.432	1.42	4.393	5.614	2.561	2.956
T (0.05)	2.306						

DAS- Days after sowing; Values are mean of 5 observations

4.6. ELUCIDATION OF ROLE OF OXIDATIVE AND SCAVENGING ENZYMES IN *P. indica*- PRIMED OKRA AGAINST BYVMV

Biochemical estimation was done separately for natural incidence, pre- and post- inoculation experiments by collecting samples at 40 days after sowing from naturally infected plants and 30 days after grafting from artificially inoculated plants. It included analysis of total chlorophyll, total soluble protein, peroxidase, polyphenol oxidase, catalase, ascorbic acid oxidase and phosphatase. Biochemical analysis of *P. indica*-primed and non-primed plants revealed increase in chlorophyll and total soluble proteins, and high activities of antioxidant enzymes in *P. indica*-colonised okra plants.

4.6.1. Total chlorophyll content

Total chlorophyll content was estimated following the method described by Arnon (1949). Virus infection drastically reduced the chlorophyll content but *P. indica*-priming significantly increased the chlorophyll content under natural and artificial infection of BYVMV.

Under natural incidence, total chlorophyll content was highest in *P. indica*-primed plants (1.32 mg g^{-1}) and least in BYVMV infected plants (0.20 mg g^{-1}). Colonisation by *P. indica* significantly enhanced total chlorophyll content (0.60 mg g^{-1}) in diseased plants. Chlorophyll b content was higher (0.47 mg g^{-1}) healthy plants but a drastic reduction in chlorophyll b content was noticed due to the virus infection (0.06 mg g^{-1}). *P. indica*- priming significantly increased (3.2 - fold) chlorophyll b content (0.19 mg g^{-1}) in naturally infected plants (Table 11).

Similar trend was observed in chlorophyll content in artificial inoculation studies. In pre-inoculation of the fungus followed by grafting of the virus, total chlorophyll and chlorophyll b contents in pre-primed diseased plants were high (0.72 mg g^{-1} and 0.24 mg g^{-1} respectively), but it was least in the non-primed diseased plants (0.26 mg g^{-1} and 0.09 mg g^{-1} respectively) (Table 15). Post priming of *P. indica* also significantly increased the total chlorophyll and chlorophyll b content over the diseased control in artificially challenged plants with BYVMV, but the increase was less compared to pre-inoculation (Table 18).

Table 11. Effect of *P. indica*-priming on chlorophyll contents in okra leaves under natural incidence of BYVMV

Treatments	Chlorophyll contents (mg g^{-1} fw)		
	Chl a	Chl b	Total Chl
Healthy	0.78 ± 0.03	0.47 ± 0.028	1.26 ± 0.002
P. indica alone	0.99 ± 0.073	0.33 ± 0.022	1.32 ± 0.056
BYVMV alone	0.13 ± 0.026	0.06 ± 0.001	0.20 ± 0.025
P. indica + BYVMV	0.41 ± 0.015	0.19 ± 0.056	0.60 ± 0.071
SE (m) ±	0.042	0.033	0.047
CD (0.05)	0.14	0.11	0.155

Values are the mean of 5 replications ± standard deviation

Table 12. Effect of *P. indica*-priming on total protein content, and peroxidase and polyphenol oxidase activities in okra leaves against natural incidence of BYVMV

Treatments	Total soluble protein (mg g^{-1} fw)	Peroxidase activity (µg min^{-1} g^{-1} fw)	Polyphenol oxidase activity (µg min^{-1} g^{-1} fw)
Healthy	3.19 ± 0.029	8.7 ± 0.363	0.84 ± 0.022
P. indica alone	4.21 ± 0.231	13.9 ± 0.260	1.16 ± 0.008
BYVMV alone	3.24 ± 0.111	8.06 ± 0.093	0.91 ± 0.022
P. indica + BYVMV	5.25 ± 0.225	18.63 ± 0.180	2.97 ± 0.096
SE (m) ±	0.171	0.245	0.051
CD (0.05)	0.567	0.812	0.168

Values are the mean of 5 replications ± standard deviation

Table 13. Effect of *P. indica*-priming on catalase, ascorbic acid oxidase and phosphatase activities in okra leaves under natural incidence of BYVMV

Treatments	Catalase (enzyme units min^{-1} g^{-1} fw)	Ascorbic acid oxidase (enzyme units min^{-1} g^{-1} fw)	Phosphatase (*m* moles of *p*-nitrophenol released min^{-1} g^{-1} fw)
Healthy	253.54 ± 7.271	0.22 ± 0.006	69.07 ± 0.117
P. indica alone	306.92 ± 16.428	0.31 ± 0.005	71.24 ±1.176
BYVMV alone	258.55 ± 8.667	0.23 ± 0.001	65.11 ± 0.889
P. indica + BYVMV	488.74 ± 31.693	0.47 ± 0.001	72.71 ± 0.444
SE (m) ±	18.724	0.004	0.772
CD (0.05)	62.009	0.013	2.557

Values are the mean of 5 replications ± standard deviation

Table 14. Effect of *P. indica*-priming against BYVMV in okra on graft transmission

Treatments	Days for symptom appearance	Vulnerability index
Healthy (control)	0	0
P. indica alone	0	0
BYVMV alone	11-15	74.00
P. indica +BYVMV (pre-inoculation)	12-16	32.50
BYVMV+ *P. indica* (post-inoculation)	11-15	56.00

Table 15. Effect of *P. indica*-priming (pre-inoculation) on chlorophyll contents in okra leaves against BYVMV on graft transmission

Treatments	Chlorophyll content (mg g^{-1}fw)		
	Chl a	Chl b	Total Chl
Healthy	0.71 ± 0.119	0.39 ± 0.049	1.095 ± 0.10
P. indica alone	0.70 ± 0.053	0.50 ± 0.009	1.202 ± 0.05
BYVMV alone	0.17 ± 0.019	0.09 ± 0.006	0.26 ± 0.025
P. indica + BYVMV	0.47 ± 0.009	0.24 ± 0.042	0.72 ± 0.04
SE (m) ±	0.066	0.033	0.0613
CD (0.05)	0.219	0.109	0.204

Values are the mean of 5 replications ± standard deviation

Table 16. Effect of *P. indica*-priming (pre-inoculation) on total protein content, and peroxidase and polyphenol oxidase activities in okra leaves against BYVMV on graft transmission

Treatments	Total soluble protein (mg g^{-1} fw)	Peroxidase activity (µg $min^{-1}g^{-1}$ fw)	Polyphenol oxidase activity (µg $min^{-1}g^{-1}$ fw)
Healthy	1.58 ± 0.064	9.44 ± 0.509	0.95 ± 0.096
P. indica alone	2.53 ± 0.029	15.92 ± 0.355	1.24 ± 0.073
BYVMV alone	1.71 ± 0.018	10.62 ± 0.263	1.05 ± 0.038
P. indica + BYVMV	4.23 ± 0.139	22.10 ± 0.279	2.92 ± 0.077
SE (m) ±	0.036	0.365	0.074
CD (0.05)	0.120	1.208	0.245

Values are the mean of 5 replications ± standard deviation

4.6.2. Total soluble protein

Total soluble protein was estimated by protocol described by Bradford (1976) and results are presented in Table 12, Table 16 and Table 19. Total soluble protein content was significantly increased due to *P. indica* priming under natural incidence of BYVMV. In healthy plants the protein content was 3.19 (mg g^{-1}) while it increased (4.21 mg g^{-1}) in *P. indica* colonised plants. Under natural incidence of BYVMV, protein content was highest *in P. indica*-primed diseased plants (5.25 mg g^{-1}) while it was least in non-primed diseased plants 3.24 (mg g^{-1}) (Table 12).

Okra plants pre-inoculated with the fungus followed by graft inoculation of the virus showed highest soluble protein content (4.23 mg g^{-1}) compared to the diseased control (1.71 mg g^{-1}) (Table 16). Plants challenged with BYVMV followed by post-inoculation of the fungus also showed high protein content (4.03 mg g^{-1}) compared to the virus inoculated control (1.82 mg g^{-1}) (Table 19).

4.6.3. Peroxidase

Peroxidase activity was determined by a protocol described by Srivastava (1987). Natural incidence of BYVMV resulted in slight decrease in the peroxidase activity (8.06 µg min^{-1} g^{-1}) compared to the healthy plants (8.7 µg min^{-1} g^{-1}). But *P. indica* colonization significantly increased peroxidase activity (18.63 µg min^{-1} g^{-1}) and the highest peroxidase activity being expressed by *P. indica*-primed naturally infected plants with the virus (18.63 µg min^{-1} g^{-1}) (Table 12).

Pre-treatment of *P. indica* followed by artificial infection of the virus by grafting also significantly enhanced the activity of peroxidase (22.10 µg min^{-1} g^{-1}) while it was less in non-colonised plants challenged with BYVMV (10.62 µg min^{-1} g^{-1}) (Table 16). Peroxidase activity in post-priming of *P. indica* in artificially challenged plants with BYVMV was also higher compared to the non-primed diseased plants but lower than the pre-treatment of the fungus (Table 19).

Table 17. Effect of *P. indica*-priming (pre-inoculation) on catalase, ascorbic acid oxidase and phosphatase activities in okra leaves against BYVMV on graft transmission

Treatments	Catalase (enzyme units min^{-1} g^{-1} fw)	Ascorbic acid oxidase (enzyme units min^{-1} g^{-1} fw)	Phosphatase (*m* moles of *p*-nitrophenol released min^{-1} g^{-1} fw)
Healthy	258.54 ± 4.413	0.22 ± 0.003	68.67 ± 0.400
P. indica alone	311.93 ± 7.644	0.28 ± 0.009	75.56 ± 0.445
BYVMV alone	268.56 ± 4.412	0.23 ± 0.015	52.89 ± 0.889
P. indica + BYVMV	507.09 ± 13.028	0.42 ± 0.005	69.78 ± 2.756
SE (m) ±	8.172	0.009	1.478
CD (0.05)	27.063	0.030	4.896

Values are the mean of 5 replications ± standard deviation

4.6.4. Poly phenol oxidase

Poly phenol oxidase (PPO) activity was assessed by following the procedure described by Mayer *et al.* (1965) and following results were observed. Under natural incidence of BYVMV, PPO activity was less (0.91 µg min^{-1} g^{-1}) but priming of *P. indica* significantly enhanced PPO activity in the naturally infected plants 2.97 (µg min^{-1} g^{-1}) (Table 12).

PPO activity was significantly higher in pre-primed plants with *P. indica* and artificially challenged by the virus (2.92 µg min^{-1} g^{-1}), compared to the non- primed virus inoculated plants (1.05 µg min^{-1} g^{-1}) (Table 16). Plants that were post-inoculated with the fungus after graft transmission of the virus exhibited maximum activity of PPO (1.88 µg min^{-1} g^{-1}) compared to the non-primed virus inoculated control (0.85 µg min^{-1} g^{-1}) (Table 19), but the increase in PPO activity was not high as in case of pre-inoculation of the fungus.

4.6.5. Catalase

Catalase activity was determined by procedure described by Luck (1974). In case of the natural infection studies, least catalase activity was shown by healthy plants (253.54 EU min^{-1} g^{-1}) followed by the virus infected plants (258.55 EU min^{-1} g^{-1}); but *P. indica*-priming significantly increased the catalase activity (306.92 EU min^{-1} g^{-1}), and the highest catalase activity being expressed by *P. indica* primed naturally infected plants (488.74 EU min^{-1} g^{-1}) (Table 13).

There was a drastic increase in catalase activity (507.09 EU min^{-1} g^{-1}) in pre-priming of *P. indica* followed by artificial infection of BYVMV, compared to the non-primed virus inoculated plants (268.56 EU min^{-1} g^{-1}) (Table 20). In post- inoculation of *P. indica* after the graft infection, highest catalase activity (427.03 EU min^{-1} g^{-1}) was observed in post-primed plants challenged with BYVMV and least in the virus inoculated plants (270.22 EU min^{-1} g^{-1}) which was on par with healthy plants (263.55 EU min^{-1} g^{-1}) (Table 20).

Table 18. Effect of *P. indica*-priming (post-inoculation) on chlorophyll content in okra leaves against BYVMV on graft transmission

Treatments	Chlorophyll content (mg g^{-1} fw)		
	A	b	Total
Healthy	0.79 ± 0.03	0.32 ± 0.033	1.116 ± 0.063
P. indica alone	0.81 ± 0.047	0.44 ± 0.028	1.25 ± 0.060
BYVMV alone	0.17 ± 0.017	0.08 ± 0.005	0.25 ± 0.021
BYVMV + *P. indica*	0.30 ± 0.061	0.25 ± 0.082	0.55 ± 0.035
SE (m) ±	0.042	0.046	0.048
CD (0.05)	0.140	0.153	0.158

Values are the mean of 5 replications ± standard deviation

Table 19. Effect of *P. indica*-priming (post-inoculation) on total protein content, and peroxidase and polyphenol oxidase activities in okra leaves against BYVMV on graft transmission

Treatments	Total soluble protein (mg g^{-1} fw)	Peroxidase activity (min^{-1} g^{-1} fw)	Polyphenol oxidase activity (min^{-1} g^{-1} fw)
Healthy	1.64 ± 0.024	9.27 ± 0.404	0.75 ± 0.014
P. indica alone	2.95 ± 0.029	14.00 ± 0.133	1.13 ± 0.004
BYVMV alone	1.82 ± 0.024	10.74 ± 0.460	0.85 ± 0.043
BYVMV+ *P. indica*	4.03 ± 0.047	16.8 ± 0.263	1.88 ± 0.051
SE (m) ±	0.032	0.340	0.034
CD (0.05)	0.107	1.126	0.113

Values are the mean of 5 replications ± standard deviation

4.6.6. Ascorbic acid oxidase

Ascorbic acid oxidase activity in *P. indica*-primed plants under the natural and artificial incidence of BYVMV was determined by protocol developed by Oberbacher and Vines, (1953) and results are represented in Table 13, table 17 and Table 20.

Priming of okra plants with *P. indica* significantly increased the ascorbic acid oxidase activity (0.31 EU min^{-1} g^{-1}) compared to healthy plants (0.22 EU min^{-1} g^{-1}). Marked increase in ascorbic acid oxidase activity was observed *P. indica* pre-treated okra plants (0.47 EU min^{-1} g^{-1}) challenged by BYVMV compared to the non-primed virus infected plants (0.23 EU min^{-1} g^{-1}) (Table 13). Plants pre- inoculated with fungus followed by grafting exhibited highest activity of ascorbic acid oxidase (0.42 EU min^{-1} g^{-1}) compared to the non-primed virus inoculated control plants (0.23 EU min^{-1} g^{-1}) (Table 17). Plants challenged with BYVMV followed by post inoculation of the fungus also showed high ascorbic acid oxidase activity (0.38 EU min^{-1} g^{-1}) compared to the diseased control (0.22 EU min^{-1} g^{-1}) (Table 20).

4.6.7. Phosphatase

Effect of *P. indica* priming on natural and artificial infection of BYVMV on phosphatase activity was analysed by the method described by Lowry *et al.* (1954). Results obtained revealed that there was a drastic reduction in phosphatase activity in the virus infected plants, but *P. indica*-priming prevented or inhibited the reduction in activity of phosphatase under natural incidence and artificial inoculation of the virus (Table 13, Table 17 and Table 20).

Table 20. Effect of *P. indica*-priming (post-inoculation) on catalase, ascorbic acid oxidase and phosphatase activities in okra leaves against BYVMV on graft transmission

Treatments	Catalase (enzyme units min^{-1} g^{-1} fw)	Ascorbic acid oxidase (enzyme units min^{-1} g^{-1} fw)	Phosphatase (*m* moles of *p*-nitrophenol released min^{-1} g^{-1} fw)
Healthy	263.55 ± 6.015	0.21 ± 0.006	69.13 ± 0.203
P. indica alone	308.59 ± 10.93	0.27 ± 0.011	71.16 ± 3.511
BYVMV alone	270.22 ± 3.337	0.22 ± 0.018	58.23 ± 2.476
BYVMV + *P. indica*	427.03 ± 17.65	0.38 ± 0.006	55.93 ± 1.591
SE (m) ±	10.938	0.011	2.293
CD (0.05)	36.225	0.038	7.594

Values are the mean of 5 replications ± standard deviation

Discussion

5. DISCUSSION

Vegetable crops play a vital role in human diet owing to their high nutritional qualities. But their production is challenged due to incidence of pest and disease. Among this, viral diseases are serious threat to the production leading to heavy crop loss. Production of okra an important vegetable crop cultivated throughout the world, is seriously affected by viral disease, bhendi yellow vein mosaic disease which is transmitted by white-fly. Current management strategies are in sufficient for management of disease. Most economic method is use of resistant varieties; but resistance break down and emergence of new strains of the virus makes this method inefficient. Use of endophytic microorganisms that elicit plant defense mechanisms is a novel, eco-friendly approach in disease management, thus becomes an inevitable part of sustainable agriculture. *P. indica***,** a beneficial fungal root endophyte, colonises within plant roots, establishes symbiotic relationships with plants and confers benefits like plant growth and development, nutrient acquisition, increased abiotic stress tolerance, and plays a major role in management of plant diseases caused by fungi, bacteria and virus. The results of the study on evaluation of *P. indica* in management of BYVMV are discussed in this chapter.

5.1 COLLECTION OF BYVMV INFECTED OKRA

A survey was conducted to study the occurrence of bhendi yellow vein disease in okra fields in three different locations of Thiruvananthapuram district *viz.,* Vellayani, Pappanchani and Palappur during July 2018 – March 2020. Disease incidence and severity was assessed based on the symptoms observed in the field. Severe infection of BYVMD was noticed in all surveyed locations with disease incidence ranging from 67 to 100 per cent. Highest D. I. of 100 percent was recorded from Palappur in okra variety Kiran with maximum V. I. of 71.07. Survey conducted by Naveen (2018) for the collection of *Geminivirus* infected okra in three thaluks of Thiruvananthapuram district revealed that highest disease incidence of 100 per cent in Chirayinkeezhu taluk with V. I. of 78.42. High disease incidence is attributed to the season of cultivation and the environmental conditions. Here crops were sown in summer season when the population of white fly is high. Similar results were obtained by Ghevariya and Mahatma (2017) who reported high incidence of the disease ranging

from 10-50 per cent in the different field of Navsari region of Gujarat during late winter to summer during 2015. Basheer (2019) conducted survey in Thiruvananthapuram and Kollam districts of Kerala during summer 2018 revealed high disease incidence (D. I.) in all surveyed locations; cent percent D. I. was reported in Vellayani, Neyyattinkara and Chirayinkeezhu areas of Thiruvannanthapuram district with V. I. of more than 65.00 in all these locations. High temperature and relative humidity favour increase in white fly population that transmit BYVMV (Mubeen *et al.*, 2017).

Apart from this, stage of infection, crop variety, strain of virus and management strategies adopted by farmers also affects the incidence of the disease (Strange and Scott, 2005). Infection at early stages of crop led to severe yield reduction and crop loss (Fajinmi and Fajinmi, 2010). All the varieties under survey shown to be susceptible to the disease, but in varying degrees. VarshaUphar which was earlier resistant to BYVMV, also showed severe infection. This was in accordance with Basheer (2019) who reported 100 percent D. I and V. I of 65.40 in var. Varsha uphar at Vellayani region. It was observed that closer spacing was adopted by farmers, and presence of weeds like *Ageratum conyzoides* and *Croton sparsiflorus* that act as collateral hosts were also noticed in all surveyed fields. Naveen (2018) reported occurrence of 19 weed species that exhibited vein clearing symptoms in surveyed okra fields that act as collateral host for the dissemination of disease.

5.2 SYMPTOMATOLOGY

Under field condition, symptoms of BYVMD depend on stage of crop being infected. In surveyed locations, various symptoms including initial vein clearing, mottling, in early stage and reduction of leaf size, stunted growth, bleached, hardened and deformed fruits in later stage of crop were observed. Apart from these, vein thickening, distorted leaves with slight curling and vein clearing on flower buds was also observed. Similar symptoms were observed by Ghevariya and Mahatma (2017) where initial symptoms on young leaves included vein clearing near leaf margin and mottled appearance and in severe infection the entire leaf curled inside, petiole bend, entire plants turns yellow and become dwarf with reduced flowering and fruit set, production of malformed and bleached fruits. Symptoms observed by Jose and Usha

(2003) include vein twisting, petiole bending, upward curling and stunted plant growth, in addition to vein clearing and complete yellowing. Characteristic symptoms of BYVMD include interwoven network of yellow veins surrounded by green tissues, later the entire leaf turns completely yellow or cream coloured, plants become stunted, production of pale-yellow coloured, small, malformed, and tough textured fruits (Singh 1990; Sanwal *et al.*, 2016).

5.3. MOLECULAR DETECTION OF BYVMV

Visual observation of symptoms caused by virus is a detection method confirming presence of virus. Immunological or serological detection using ELISA is also widely employed for detection of plant viruses but more reliable, sensitive and accurate method is molecular or PCR-based detection using specific primers (Jeong *et al.*, 2014).

For carrying out molecular detection of BYVMV, total plant DNA was isolated using DNeasy plant mini kit (QIAGEN: Cat. No. 69104) and presence of DNA was confirmed by performing agar gel electrophoresis followed by visualization in UV trans illuminator system (Bio-Rad).

The quality and quantity of isolated DNA was assessed in biospectrophotometer by taking absorbance values at 260 nm and 280 nm. High quality DNA should show a 260/280 value of 1.8. But the results revealed that the quality of isolated DNA was less and ranged from 1.21 to 1.31 in Palappur and Vellayani isolate of BYVMV respectively. Quality was less due to high mucilage content in okra that leads to protein or polysaccharide contamination in the extracted DNA. Similar results were obtained by Basheer (2019) where the quality of isolated DNA was low and it ranged from 1.11 to 1.51 but this did not interfere with quantity of DNA. Kumar (2018) isolated BYVMV mucilage free DNA by using protocol described by Porebski *et al.*, (1997) and could yield high quality DNA with 260/280 value of 1.8.

Molecular detection of BYVMV was done by PCR using two universal primers *viz.*, AV/AC (Wyatt and Brown, 1996) and Deng (Deng *et al.*, 1994) specific to coat protein of BYVMV (*Begomovirus*). Results revealed that all isolates of

BYVMV could produce amplicons of size 575 bp with AV/AC primer and amplicon size of 520 bp with Deng primer, thus the presence of virus was confirmed. Naveen (2018) and Basheer (2019) also done the molecular detection of BYVMV using AV/AC and Deng primers and could yield amplicons of size 575 bp and 520bp respectively. Presence of a *Begomovirus* component equivalent to DNA-A of BYVMV was identified in yellow vein infected bhendi plants by PCR-amplification using primers specific to *Begomovirus* (Jose and Usha, 2000). Kumar (2018) used degenerate primers (Deng *et al*., 1994) for PCR amplification BYVMV and could yield amplicon of size approximately 500bp.

PCR analysis of DNA isolated from pollen grains of infected samples also revealed the presence of virus in pollen. BYVMV is transmitted by whitefly; there is no previous reports regarding presence of virus in pollen grains or pollen transmission of BYVMV. But Eui-Joon *et al.* 2016 detected the presence of *Tomato yellow leaf curl virus* (*Begomovirus*) from floral tissues (stamen, pistil) and seed. BYVMV was detected from immature okra seeds (Basheer, 2019). The virus either present in floral tissues or pollen may have transmitted to the immature seeds. Further studies should be conducted to confirm this.

5.4. CO-CULTIVATION OF OKRA WITH BENEFICIAL ROOT ENDOPHYTIC FUNGUS, *Piriformospora indica*

5.4.1. Maintenance of *P. indica* culture

P. indica was maintained in Potato Dextrose Agar (PDA) medium by continuous subculturing, and also in potato dextrose broth PDB. The culture of *P. indica* was maintained on potato dextrose agar (PDA) medium at room temperature (28±2ºC) in dark (Kumar *et al*. 2011; Sharma *et al.,* 2014; Lakshmipriya, 2017; Nassimi and Taheri, 2017; Anith *et al.,* 2018; Chandran, 2019; Cheng *et al*., 2020).

5.4.2. Co-cultivation of *P. indica* with okra

Modified PNM medium was used for *in-vitro* co-cultivation of *P. indica* with okra, which supports both fungal and plant growth. Surface sterilized seeds were placed in PNM medium with fully grown *P. indica* mycelium. Similar procedure was adopted by Chandran (2019) for *invitro* co-cultivation of cowpea with *P. indica*. *In-*

vitro co-cultivation of *A. thaliana* was done in PNM medium, where 12 days old seedlings from MS media were transferred to PNM plates containing *P. indica* (Johnson *et al.,* 2011; Vahabi *et al.,* 2016). Tarte *et al.* (2019) carried *in vitro* co-culture as described by Johnson *et al.* (2011) where 12 days old seedlings were transferred to modified PNM medium containing previously cultured *P. indica*.

Okra seedlings co-cultivated with *P. indica* was examined for colonisation in roots; Fungal mycelium was observed on root surface after 10 days of co-cultivation. Chlamydospores were seen on root surface after 15 days of co-cultivation and colonisation inside root initiated twenty days after co-cultivation. Massive colonisation with fully developed chlamydospores in chains was observed within the root cells after 25 days of co- cultivation. Chandran (2019) reported that in cowpea, fungal chlamydospores were observed on the roots after 7 days of co-cultivation, chlamydospore production inside the root was initiated after 10 days of co-cultivation and mature chlamydospores were observed after 14 days of co- cultivation. In groundnut, mature pear shaped chlamydospore within was observed after 45 days of *in vitro* co-cultivation (Tarte *et al.*,2019).

Growth promotion of okra seedlings was assessed at different intervals of *in vitro* co- cultivation with *P. indica*. *P. indica* colonised plants showed 29 and 50 per cent increase in the shoot and root biomass respectively over the un- colonised plants after 20 days of co- cultivation. Root length and number of secondary roots were also increased due to colonisation. Longer root was recorded for *P. indica* colonised plants while it was shorter non- colonised plants after 20 days of co- cultivation. Colonisation of *P. indica* resulted in 49 per cent increase in secondary roots formation compared to control plants. *P. indica*-colonization in Anthurium seedlings resulted in significant increase in growth, number of branches, height, leaf area, root length and biomass compared to uncolonized seedlings after 30 days of co-cultivation (Lin *et al*., 2019). Tarte *et al.* (2019) reported that *in vitro* co-culture of groundnut seedlings with *P. indica* significantly enhanced shoot length, root length and total biomass; there was 47 per cent increase in root length, 6- fold increase in fresh weight and 1.2- fold increase in dry weight after 45 days of co- culture.

In vivo co-cultivation of *P. indica* was done in potting mixture mixed with 1 percent (w/w) mycelium of *P. indica.* Colonisation of *P. indica* was observed inside the roots after 25 days of co-cultivation. But colonisation was earlier in cowpea; intracellular chlamydospores were found after 14 days of co-culture (Alex, 2017; Chandran, 2019). Tarte *et al*. (2019) observed *P. indica* chlamydospores in root cells of groundnut after 20 days of co-culture. Thus, colonisation of *P. indica* depends on crop, growth conditions, duration of co-cultivation etc.

5.5. EVALUATION OF *P. indica*-PRIMED OKRA SEEDLINGS AND PLANTS AGAINST BYVMV

Effect of *P. indica* priming against the natural incidence of BYVMV and artificial inoculation by grafting was evaluated in okra var. Salkeeerthi, which is susceptible to BYVMV and the results revealed that *P. indica*- priming reduced the D. I. by 57 per cent and V. I. by 56 per cent over control plants after 40 days of sowing. Both pre- and post-inoculation of the fungus in artificially infected okra plants also reduced the V. I. by 56 per cent and 24 per cent respectively.

Wang *et al*. (2015) showed that there was significant reduction in *Tomato yellow leaf curl virus* (TYLCV) (*Begomovirus*) infection in *P. indica* colonised susceptible variety of tomato; TYLCV incidence and disease index of non-inoculated plants were 33 per cent and 1.72 respectively but *P. indica* colonised plants showed disease incidence and index of 7 per cent and 0.47, respectively.

Alex (2017) reported that pre-treatment of *P. indica* reduced V. I by 53 per cent and 46 percent over control after 15 and 30 days of artificial inoculation of *Cowpea mosaic virus* respectively. Study conducted by Chandran (2019) revealed that there was 71 per cent reduction in V. I. in cowpea plants pre-inoculated with *P. indica* followed by artificial inoculation of *Blackeye cowpea mosaic virus* (BlCMV). *P. indica* root colonization in local lesion host of BlCMV, *Chenopodium amaranticolor* plants significantly reduced the local lesions developed by BlCMV with the per cent inhibition ranging from 60 to 73 over diseased control. Decrease in disease incidence and severity in *P. indica* colonised plant is due to induction of systemic resistance.

Stolyarchuk *et al*., 2009 observed that Arbuscular Mycorrhizal Fungi (AMF) *Rhizophagus irregularis,* colonisation in tobacco and cucumber plants followed by

Tobacco mosaic virus (TMV) and *Cucumber green mottle mosaic virus* (CGMMV) infection, respectively, reduced disease symptoms and virus titre. AMF fungi, *Funneliformis mosseae* colonization in tomato plants reduced disease severity and viral DNA concentrations in mycorrhiza colonised virus inoculated plants compared to in virus inoculated plants (Maffei *et al*., 2014).

Growth parameters were significantly higher in *P. indica* primed plants under natural incidence of BYVMV plants than in control plants. Plant height was 20 per cent higher in *P. indica* colonised plants than in un- colonised diseased plants. *P. indica* primed plants exhibited larger leaf area more than 25 per cent compared to non-primed plants but there was no significant difference in number of leaves. There was 48 per cent and 62 per cent increase in root and shoot fresh weight respectively in *P. indica*-colonised plants over control. Thus, *P. indica* promoted growth in okra plants under stress conditions. The fungus acts as biofertilizer, helps in better absorption and assimilation of plant nutrients especially phosphorus, and increase in photosynthetic rate (Achatz *et al*., 2010). Moreover, *P. indica* induced phytohormone production *viz.,* auxin, cytokinin in plants which was implicated by the increase in shoot and root biomass (Oelmuller *et al.,* 2009; Franken, 2012; Varma *et al.,* 2012b; Johnson *et al.,* 2014). *P. indica* enhanced the host plants` under low light conditions. Similar results were obtained by Chandran (2019) who reported that there was a 11 and 6 - fold increase in shoot and root biomass respectively in *P. indica* colonised plants challenged with BlCMV.

Patil *et al.* (2011) reported that fluorescent Pseudomonas 218(1) treated BYVMV infected okra plants showed 46, 56, 62 and 132 per cent increase in plant height, total biomass, chlorophyll content and fruit yield, respectively over the diseased control.

PCR analysis of naturally infected okra plants revealed that virus amount was less in *P. indica* colonised plants compared to non- colonised diseased plants. Beneficial microbes including endophytes induced systemic resistance in plants (Van Loon *et al.,* 1998*:* Mollitor and Kogel, 2009), this may have prevented further replication or spread of virus in *P.indica* colonised plants. Fakhro *et al*. (2010) reported that there was a reduction in *Pepino mosaic virus* concentration in *P. indica*-

colonised tomato plants under high light intensity. PGPR treated okra plants showed less viral load than non-treated BYVMV infected plants (Patil *et al.,* 2011).

Once colonised in plants *P. indica* offers systemic bioprotection throughout the life time of crop. Interaction of *P. indica* with plant root lead to activation of signalling pathways which leads to changes in plant transcriptome, proteome and metabolome, and phytohormones that offer systemic effects in entire plant (Johnson *et al.,* 2011).

5.6. ELUCIDATION OF ROLE OF OXIDATIVE AND SCAVENGING ENZYMES IN *P. indica*- PRIMED OKRA AGAINST BYVMV

P. indica plays a vital role in management of disease through various mechanisms including enhancement of plant growth, triggered expression of defense related genes. Biochemical analysis of *P. indica*-primed and non-primed plants under natural incidence of BYVMV and artificial transmission by grafting revealed an increase in chlorophyll and total soluble proteins, and high activities of antioxidant enzymes in *P. indica* colonised plants. Plant antioxidant system is activated in *P. indica*-induced stress resistance.

Infection of okra with BYVMV led to a significant reduction in chlorophyll content in particular chlorophyll b content compared to healthy plants under natural incidence and artificial inoculation by grafting. Chlorophyll b is more sensitive to viral infection than chlorophyll a (Anuradha *et al.,* 2015). Reduction in chlorophyll content in virus infected plants may be due to destruction of chloroplast ultrastructure and pigment synthesis or increased activity of chlorophyllase enzymes or use of chloroplast synthesis proteins by virus (Zaitlin and Hull, 1987; Balachandran *et al.,* 1997; Khalil *et al*., 2014). However, colonisation of *P. indica* in infected plants significantly enhanced the chlorophyll content; there was a 3-fold increase in total chlorophyll and 3.2-fold increase in chlorophyll b content under natural incidence of BYVMV compared to diseased control. Similar trend as in case of natural incidence was also observed in chlorophyll content in *P. indica*-primed okra plants under artificial inoculation. Tanha *et al.,* (2014) demonstrated that *P. indica* colonisation in globe artichoke prevented the reduction in chlorophyll content due to drought stress. Aseel *et al*. (2019) reported that AMF- *Funneliformis mosseae, Rhizoglomus clarum*

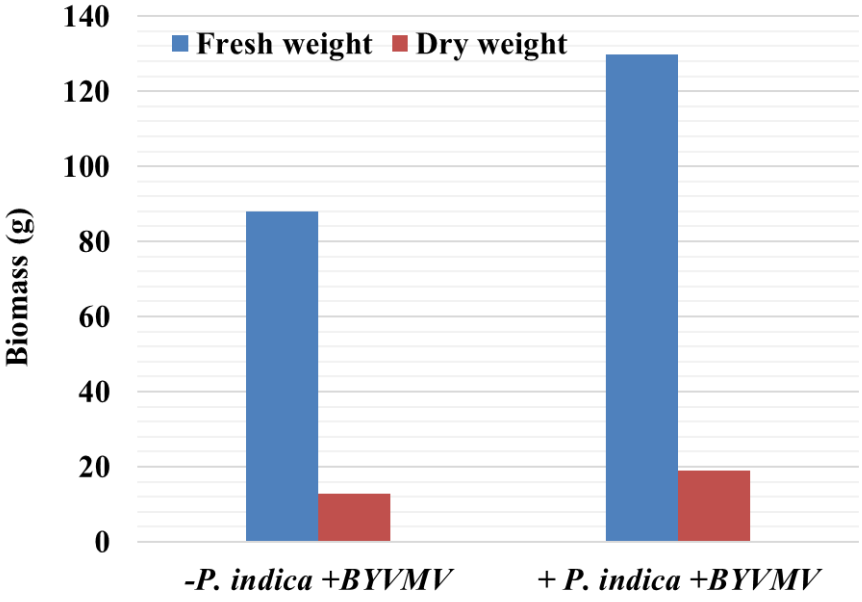


Fig. 1. Shoot biomass of *P. indica*-primed okra plants var. Salkeerthi under natural incidence of BYVMV at 40 DAS

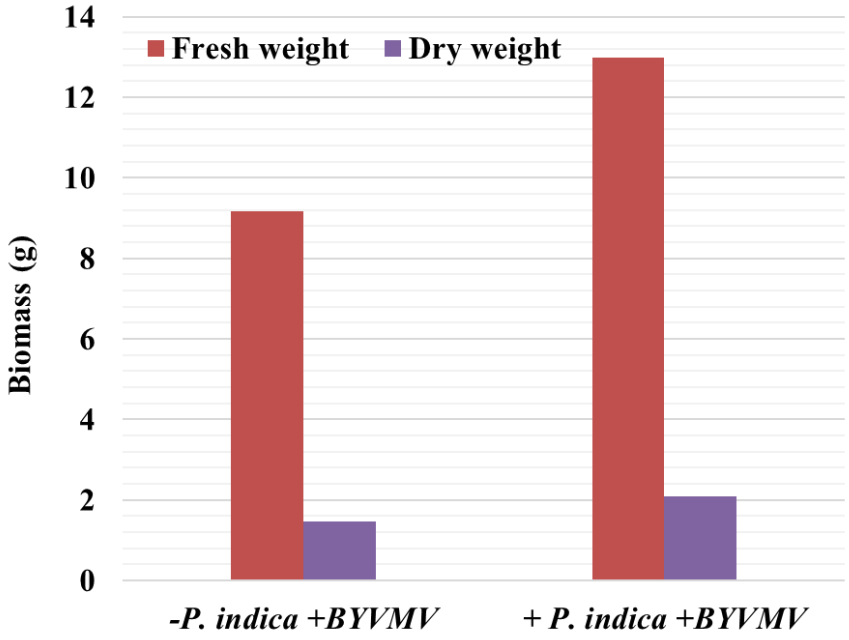


Fig. 2. Root biomass of *P. indica*-primed okra plants var. Salkeerthi under natural incidence of BYVMV at 40 DAS

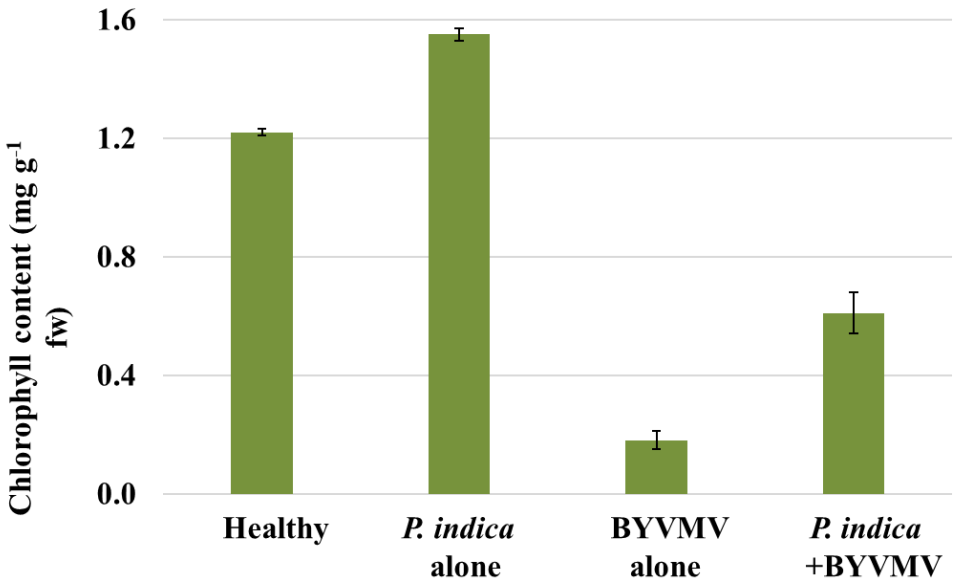


Fig. 3. Effect of *P. indica*-priming on total chlorophyll content in okra leaves under natural incidence of BYVMV at 40 DAS

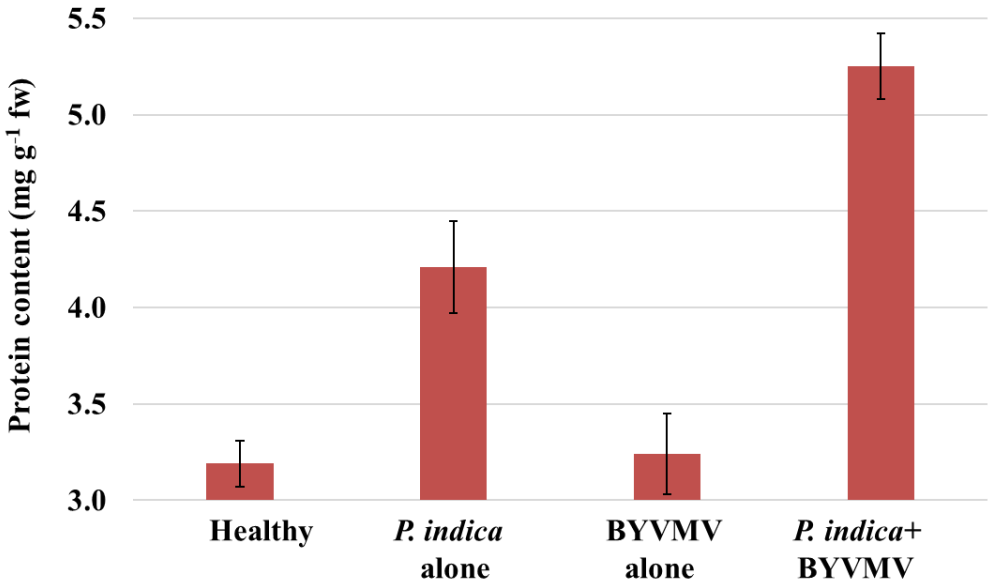


Fig. 4. Effect of *P. indica*-priming on total soluble protein in okra leaves under natural incidence of BYVMV at 40 DAS

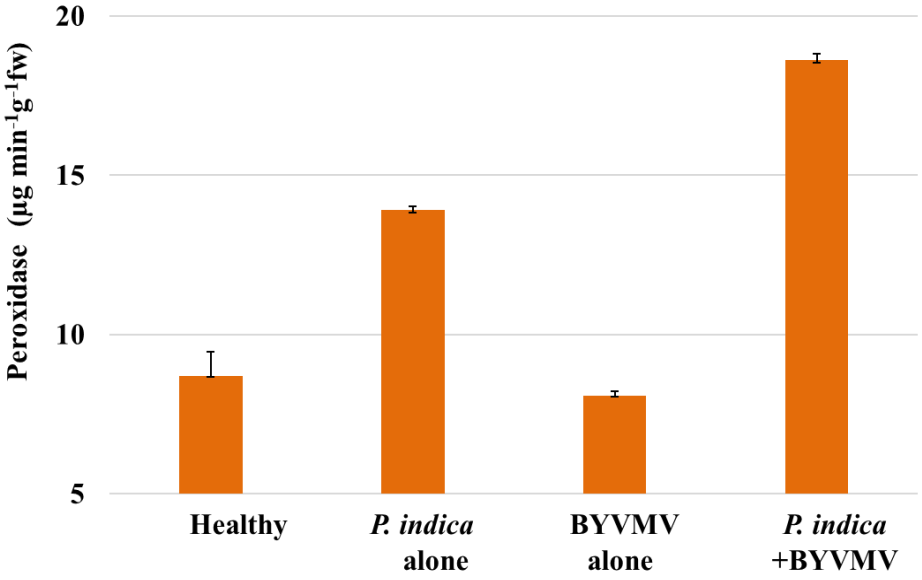


Fig. 5. Effect of *P. indica*-priming on peroxidase activity in okra leaves against natural incidence of BYVMV at 40 DAS

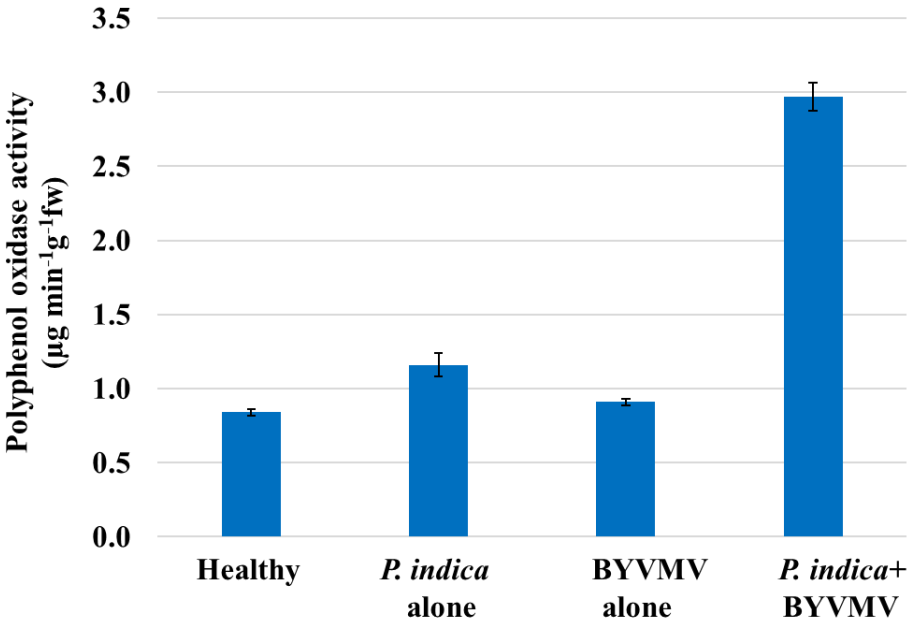


Fig. 6. Effect of *P. indica*-priming on polyphenol oxidase activity in okra leaves against natural incidence of BYVMV at 40 DAS

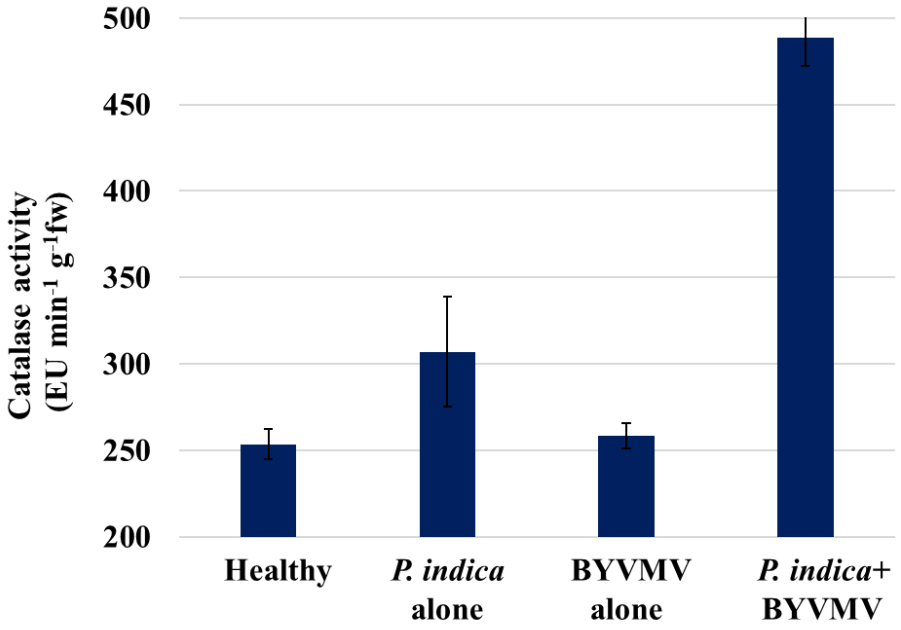


Fig. 7. Effect of *P. indica*-priming on catalase activity in okra leaves under natural incidence of BYVMV at 40 DAS

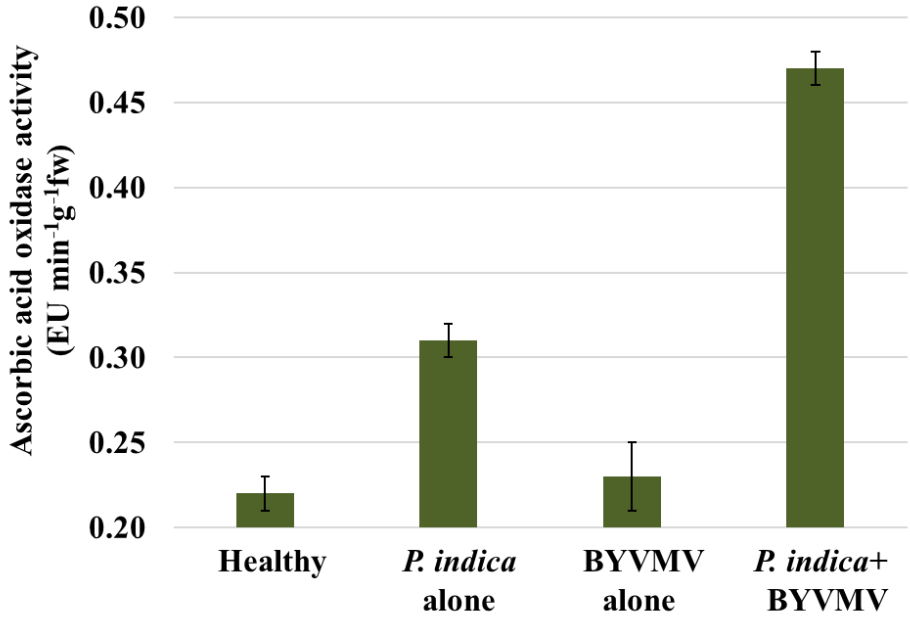


Fig. 8. Effect of *P. indica*-priming on ascorbic acid oxidase activity in okra leaves under natural incidence of BYVMV at 40 DAS

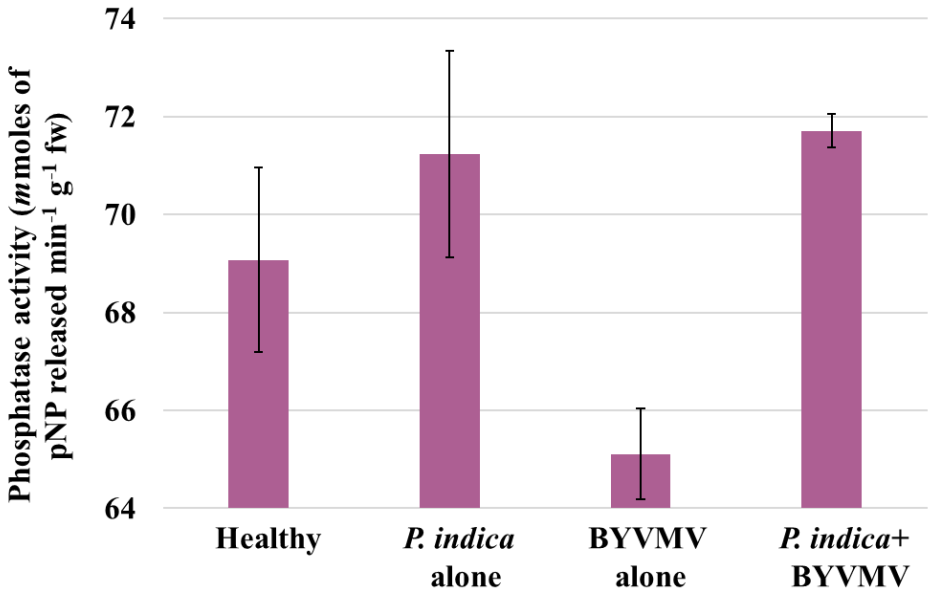


Fig. 9. Effect of *P. indica*-priming on phosphatase activity in okra leaves against natural incidence of BYVMV at 40 DAS

and *Rhizophagus aggregatus* colonisation in tomato plants enhanced photosynthetic pigments (*Chl*. a and b) content of the *Tomato mosaic virus* infected plants.

Total soluble protein content was significantly higher, a 121 per cent and 147 per cent increase was noticed in *P. indica* primed plants under natural incidence of BYVMV and in pre-inoculation of *P. indica* followed by grafting. A higher protein content in *P. indica* primed virus infected plants may be due to increase in growth and activation of host defense mechanism by production more defense related enzymes. Chandran (2019) reported that higher protein content was recorded in *P. indica* primed BlCMV infected plants 10 days after inoculation.

Enhanced activities of antioxidant enzymes like peroxidase (PO), polyphenol oxidase (PPO), catalase was observed in *P. indica*- mediated resistance against BYVMV in both natural and artificial incidence of disease. Also, in general *P. indica* colonised plants showed higher antioxidant enzyme activity compared to healthy plants. There was slight increase enzyme activity in diseased plants but the increase was not significant.

Under natural incidence of BYVMV, *P. indica* -primed plants showed 94 and 226 per cent increase in PO and PPO activity respectively. There was 48 per cent increase in activities of both catalase and ascorbic acid oxidase in *P. indica* -primed plants over the diseased control under natural incidence.

P. indica pre-primed plants challenged with BYVMV recorded maximum PO, PPO, catalase and ascorbic acid oxidase activities which were increased by 109, 176, 96 and 56 per cent respectively over the diseased control. Some studies state that activation of plant defense was associated with decrease in activity of ascorbic acid oxidase, but in contrary, present study revealed that increased activity of this enzyme in *P. indica* primed plants. There was a drastic reduction in phosphatase activity in the virus infected plants. Acid phosphatase play a crucial role in phosphorus metabolism. The enzyme catalyzes the hydrolysis of phosphatic esters resulting in release of inorganic phosphate (Duff *et al.*, 1994). Chatterjee and Gosh (2008) reported that activity of acid phosphatase was seriously impaired in mesta plants due to *Yellow vein mosaic virus* infection. In the present experiment, *P. indica*-priming could effectively

prevent or inhibit the reduction of phosphatase activity, indicating its role in *P. indica* mediated disease suppression.

The biochemical activities were significantly increased in post-inoculation of the fungus, but were not substantially as in the pre-inoculation followed by grafting of the virus, indicating effectiveness of prophylactic application of *P. indica* in disease management. Induced disease resistance is associated with the upregulation of antioxidant enzyme activities that scavenge the reactive oxygen species formed during pathogen infection, thus protecting the cell from oxidative burst.

Protection from salt stress in *P. indica* colonized plants was associated elevated the amount of antioxidant enzymes viz., catalase, ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase and glutathione reductase (Baltruschat *et al*., 2008). Kumar *et al.* (2009) observed that the *P. indica*-colonization provided protection against the root pathogen *F. verticilloides* by increased activity of the antioxidant enzymes catalase, glutathione reductase, glutathione S transferase and superoxide dismutase in maize plants; there was 23, 3.8 and 1.7 - fold increase in activity of CAT, GST and SOD respectively in colonized plants compared to un-colonized diseased plants. Sun *et al*. (2010) observed that *P. indica* colonization in Chinese cabbage resulted in increase of photosynthetic efficiency by preventing degradation of chlorophylls and thylakoid proteins under drought stress conditions by enhanced activities of peroxidase, catalase and superoxide dismutase. *P. indica* induced systemic resistance against rice sheath blight by decreasing the levels of hydrogen peroxide and by increased activity of superoxide dismutase (Nassimi and Taheri, 2017). Li *et al.,* (2017) demonstrated that enzyme activities of peroxidase and catalase was increased by 44 and 38 per cent in *P. indica* primed *Medicago truncatula* plants subjected to salt stress. *P. indica* primed cowpea plants showed increased activity of defense enzymes like peroxidase, polyphenol oxidase and conferred protection against *Black eye cowpea mosaic virus* (Alex, 2017; Chandran, 2019). The activity of the catalase was upregulated in *P. indica* inoculated rice seedlings under water stress (Tsai *et al*., 2020). AMF (*Funneliformis mosseae*) induced disease resistance against early blight in tomato by enhancing defense-related genes like *PAL, LOX, PR2, PR3, AOC, PR3* (Song *et al.,* 2015).

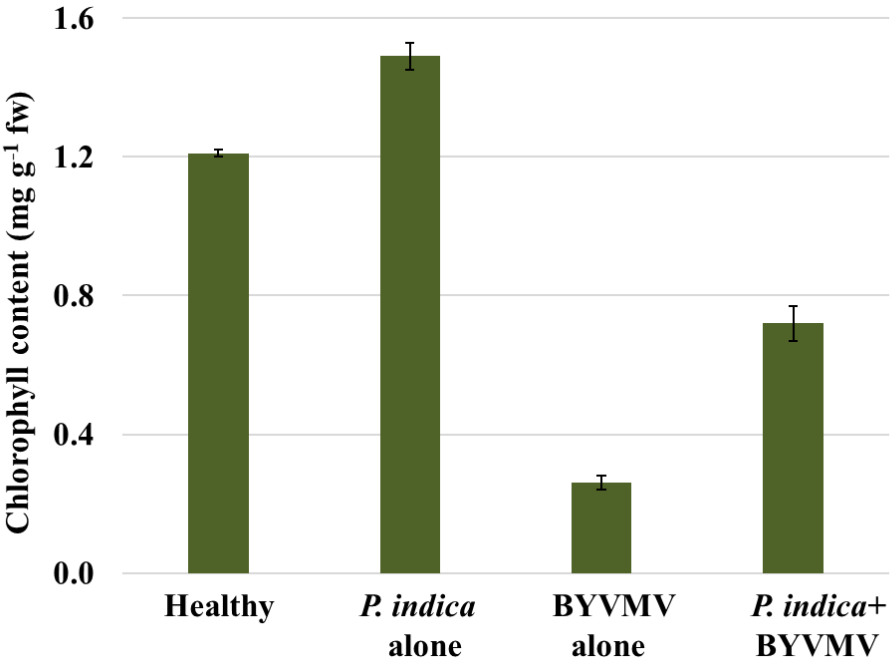


Fig. 10. Effect of *P. indica*-priming (pre-inoculation) on total chlorophyll content in okra leaves against BYVMV on graft transmission at 30 DAG

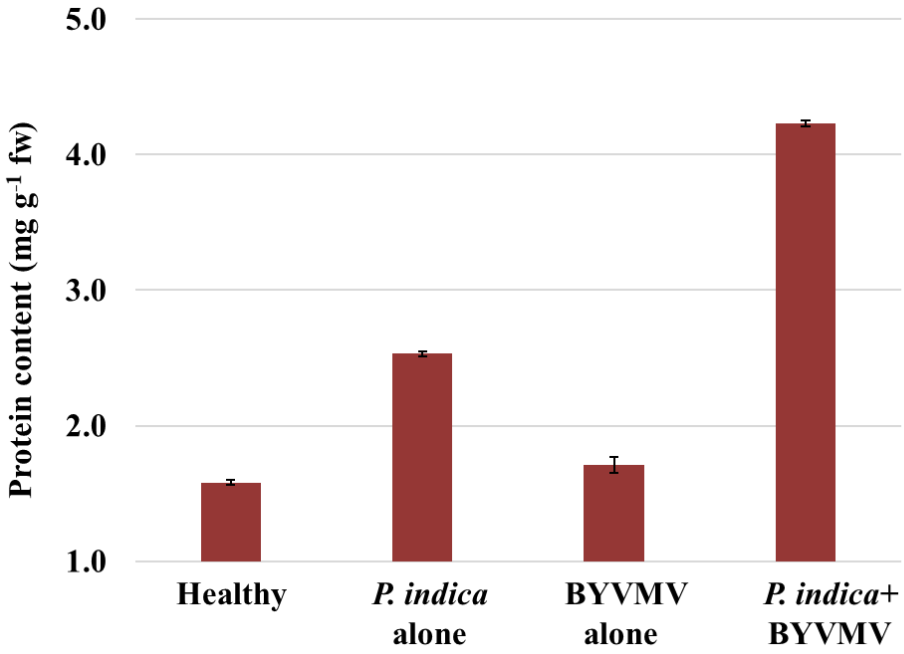


Fig. 11. Effect of *P. indica*-priming (pre-inoculation) on protein content in okra leaves against BYVMV on graft transmission at 30 DAG

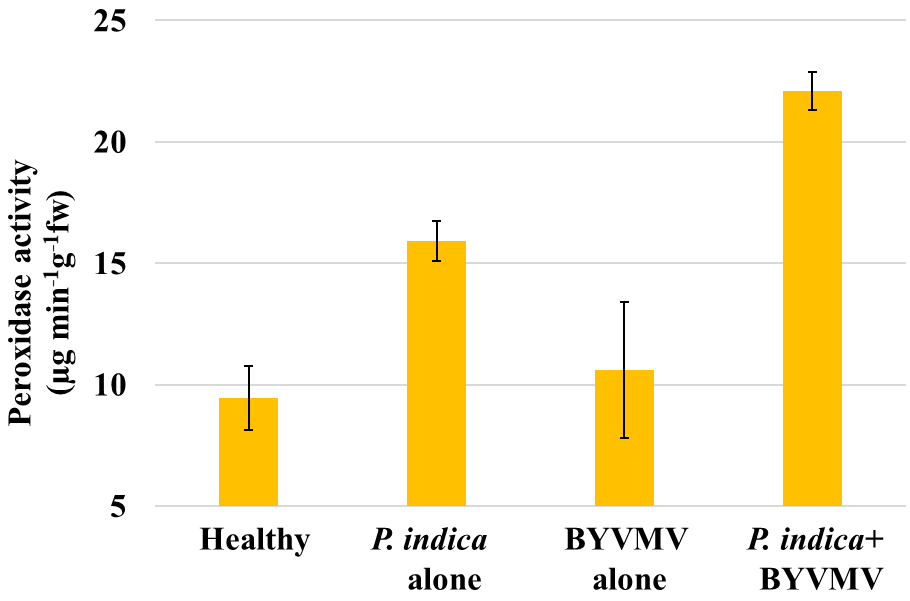


Fig. 12. Effect of *P. indica*-priming (pre-inoculation) on peroxidase activity in okra leaves against BYVMV on graft transmission at 30 DAG

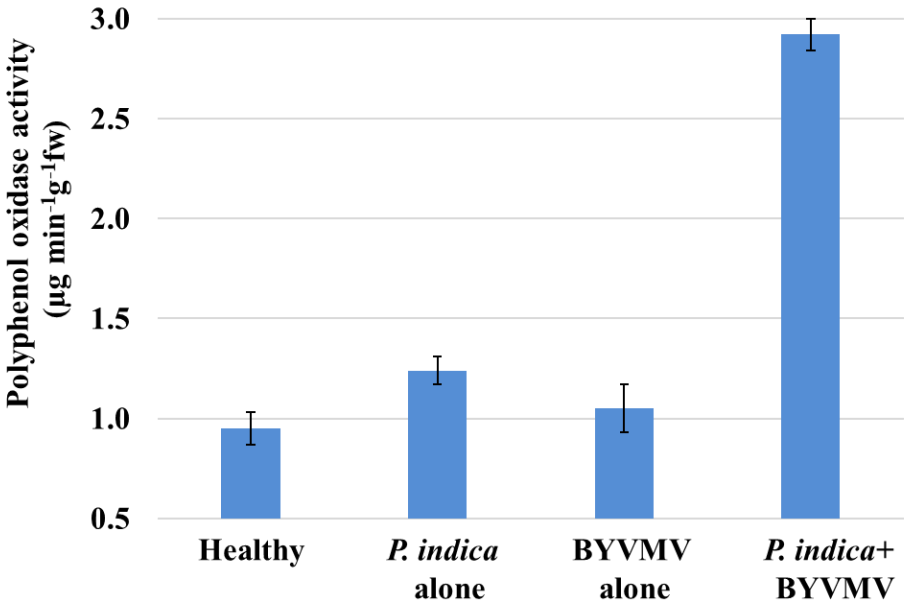


Fig. 13. Effect of *P. indica*-priming (pre-inoculation) on polyphenol oxidase activity in okra leaves against BYVMV on graft transmission at 30 DAG

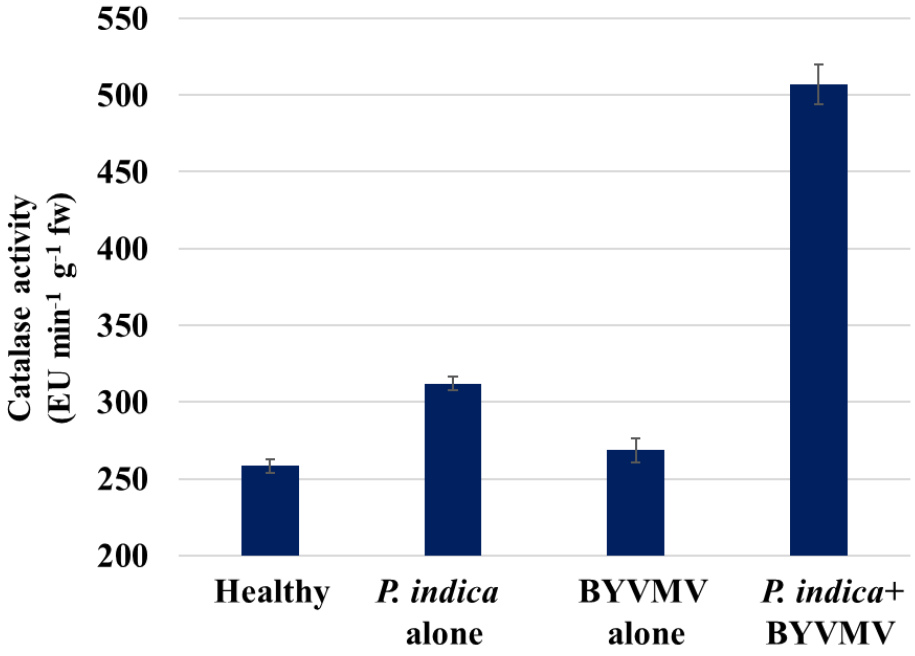


Fig. 14. Effect of *P. indica*-priming (pre-inoculation) on catalase activity in okra leaves against BYVMV on graft transmission at 30 DAG

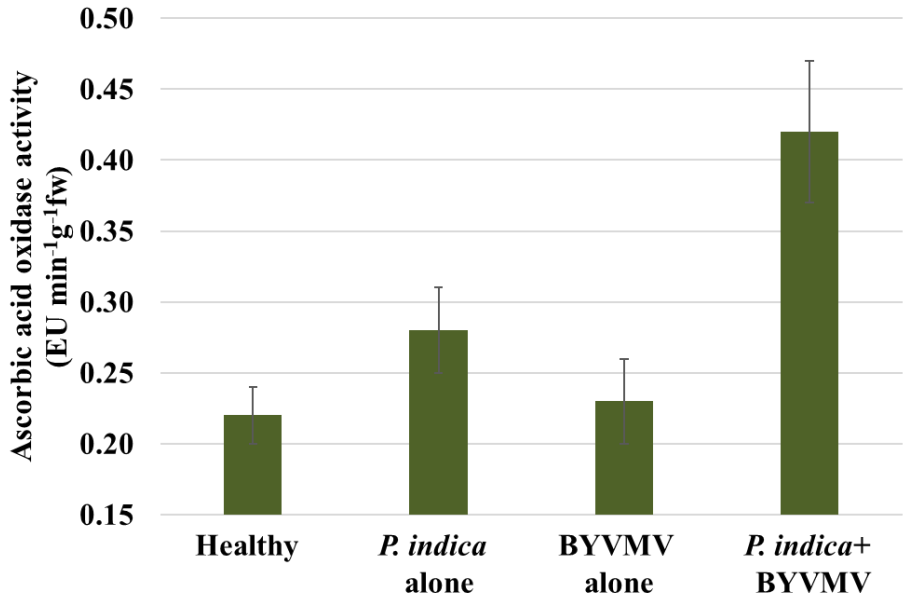


Fig. 15. Effect of *P. indica*-priming (pre-inoculation) on ascorbic acid oxidase activity in okra leaves against BYVMV on graft transmission at 30 DAG

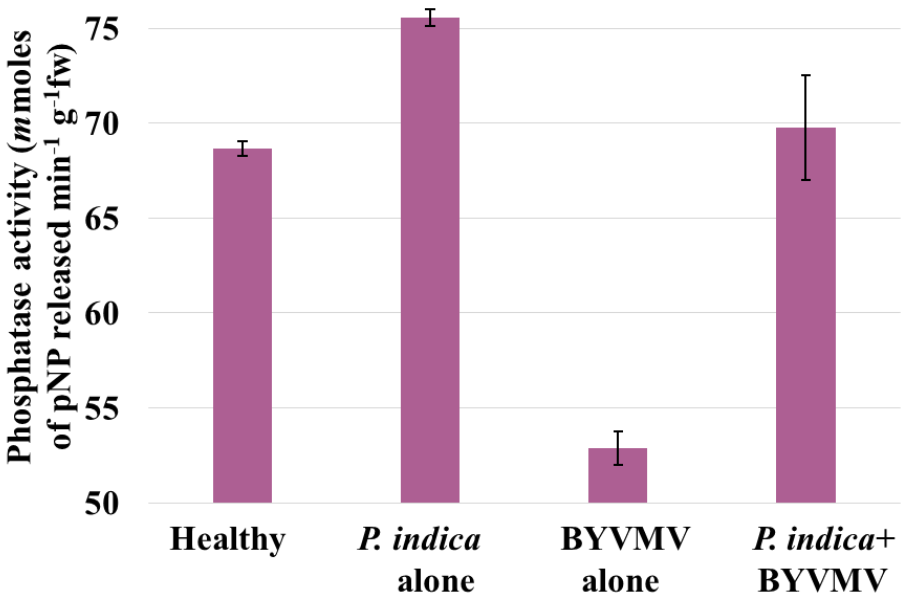


Fig. 16. Effect of *P. indica*-priming (pre-inoculation) on phosphatase activity in okra leaves against BYVMV on graft transmission at 30 DAG

Venkatesan *et al.* (2010) reported that black gram plants treated with *P. fluorescens* induced resistance against *Urd bean yellow mosaic virus* by increased activities of PO, PPO and PAL enzymes. Fluorescent Pseudomonas 218(1) treated okra plants recorded the 79.35 per cent and 47.05 per cent peroxidase activity and PAL activity respectively higher than the BYVMV infected control plants (Patil *et al.,* 2011).

Exact reason for the *P. indica* mediated resistance against viral pathogen is not clearly understood. Further studies in detail should be conducted to elucidate the role of other antioxidants, and molecular mechanisms involved in this host-endophyte-virus interaction.

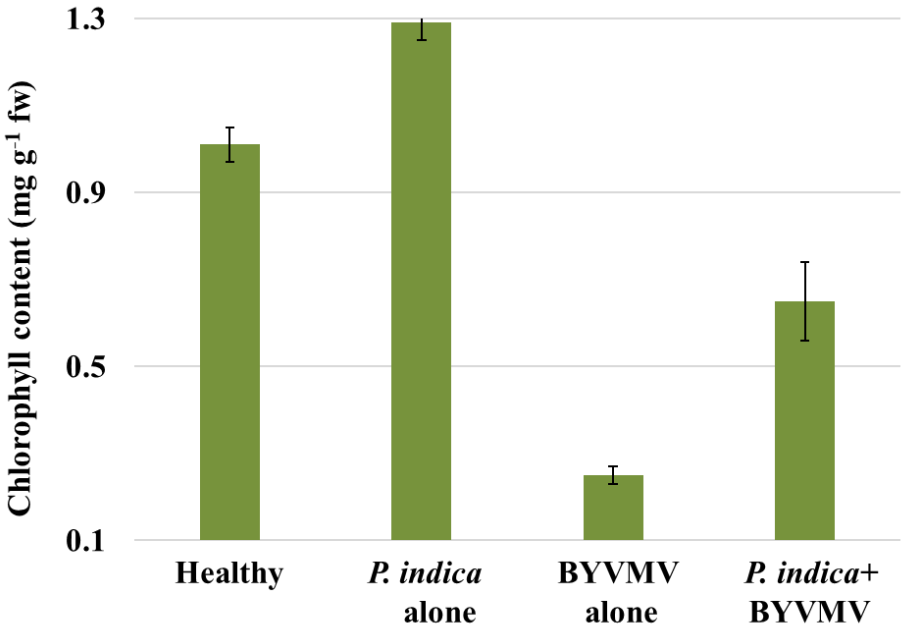


Fig. 17. Effect of *P. indica*-priming (post-inoculation) on total chlorophyll content in okra leaves against BYVMV on graft transmission at 30 DAG

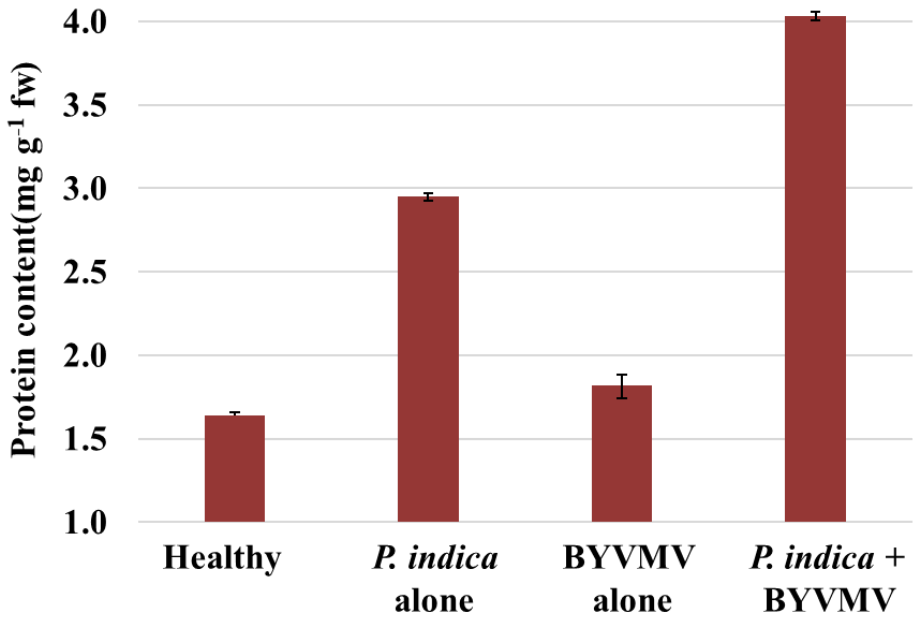


Fig. 18. Effect of *P. indica*-priming (post-inoculation) on total soluble protein content in okra leaves against BYVMV on graft transmission at 30 DAG

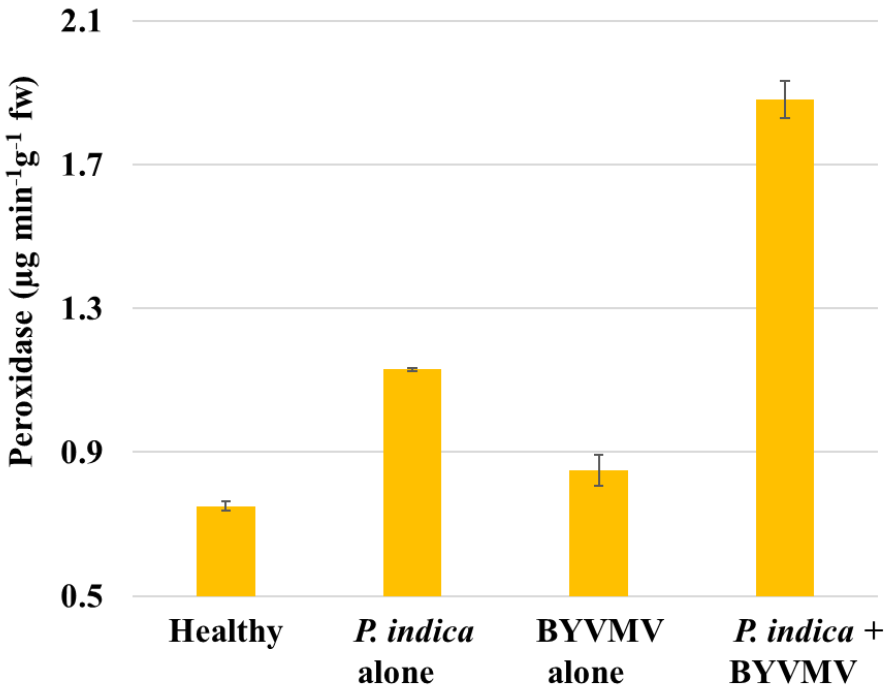


Fig. 19. Effect of *P. indica*-priming (post-inoculation) on peroxidase activity in okra leaves against BYVMV on graft transmission at 30 DAG

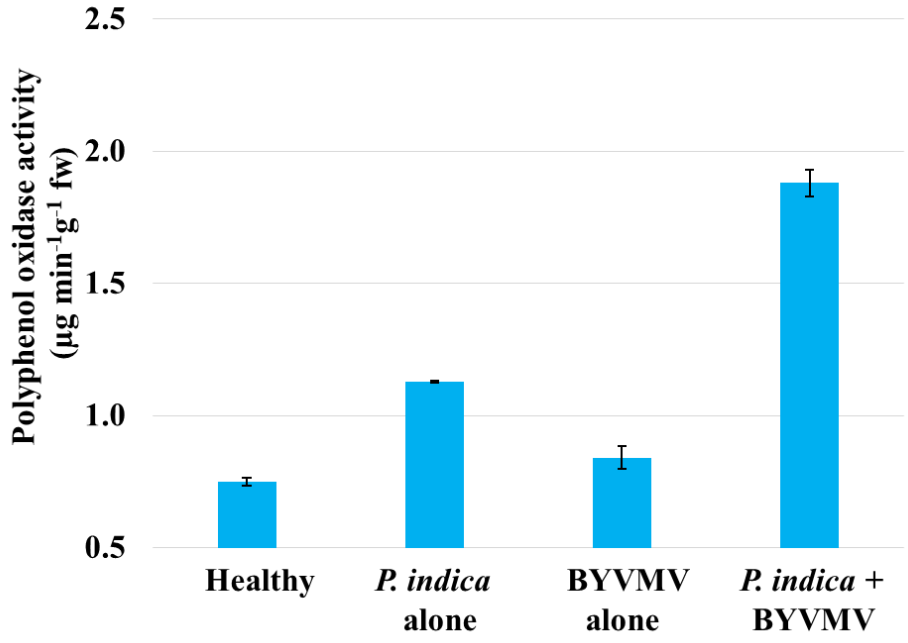


Fig. 20. Effect of *P. indica*-priming (post-inoculation) on polyphenol oxidase activity in okra leaves against BYVMV on graft transmission at 30 DAG

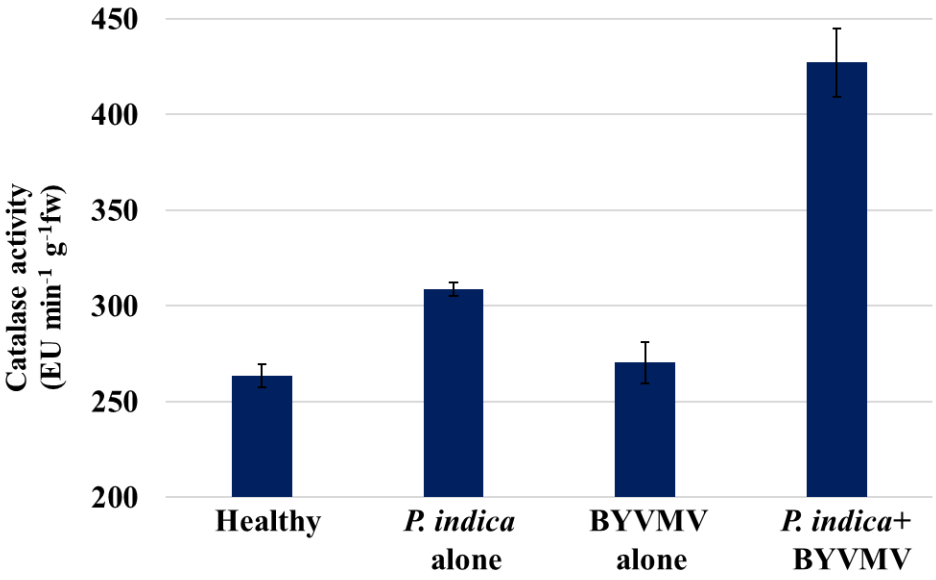


Fig. 21. Effect of *P. indica*-priming (post-inoculation) on catalase activity in okra leaves against BYVMV on graft transmission at 30 DAG

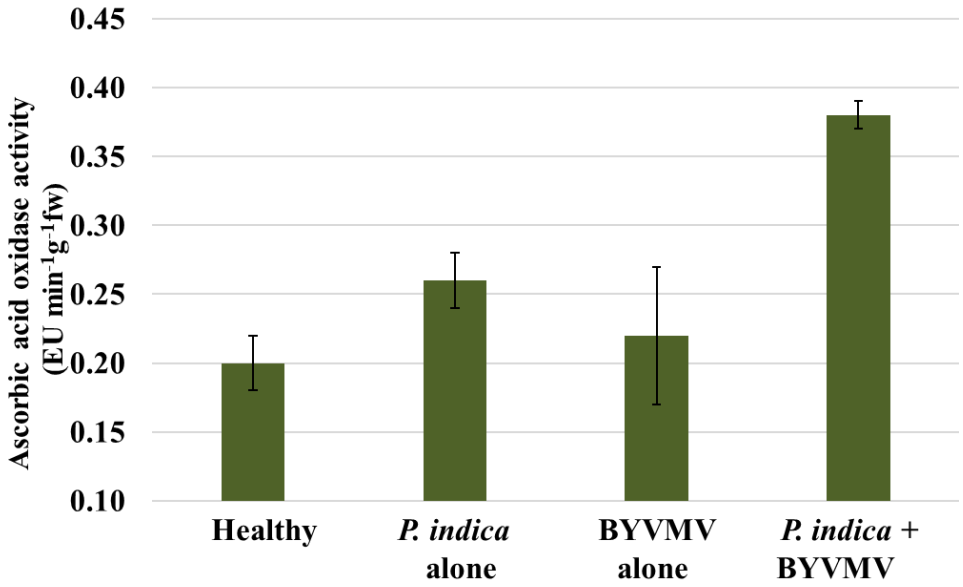


Fig. 22. Effect of *P. indica*-priming (post-inoculation) on ascorbic acid oxidase activity in okra leaves against BYVMV on graft transmission at 30 DAG

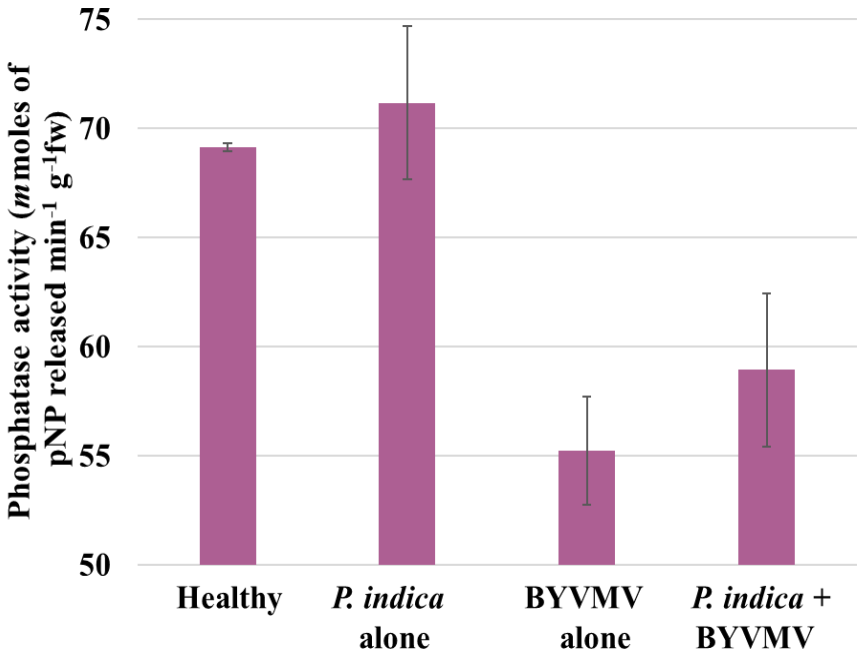


Fig. 23. Effect of *P. indica*-priming (post-inoculation) on phosphatase activity in okra leaves against BYVMV on graft transmission at 30 DAG

Summary

6. SUMMARY

The research work entitled “Management of *Bhendi yellow vein mosaic virus* using beneficial fungal root endophyte *Piriformospora indica*” was carried out in the Department of Plant Pathology, College of Agriculture, Vellayani during the period 2018-2020, to evaluate the beneficial fungal root endophyte, *P. indica* for the management of BYVMV and to elucidate the role of antioxidants in the tripartite interaction.

Survey conducted in three different locations of Thiruvananthapuram district *viz.,*Vellayani, Pappanchani and Palappur during July 2018 – March 2020, for collection BYVMV infected okra samples and assessment of the disease incidence and severity revealed that the surveyed locations were severely infected with BYVMV. The BYVMD incidence in the various surveyed locations varied from 67 to100 per cent. The highest disease incidence of cent percent was recorded from Palappur in the okra variety Kiran with maximum V. I. of 71.07. Among the surveyed locations lowest D. I. and V. I. of 67 per cent and 32.60 respectively, was recorded from Vellayani in the okra variety Anjitha.

Symptoms of BYVMD observed in different okra varieties in survey locations include mottling and vein clearing of leaves, reduction of leaf size, stunted growth, and bleached hardened and malformed fruits. In addition to these symptoms, vein thickening, and distorted leaves with slight curling were observed in Pappanchani and Palappur. Apart from this, vein clearing on flower buds was observed in Palappur.

Total DNA of infected plant samples were isolated; and quantity and quality of DNA were assessed. Molecular detection of collected diseased okra samples was done by PCR using two universal primers (AV/AC and Deng) specific to coat protein of BYVMV (*Begomovirus*). All the isolates yielded amplicons of size 575 bp with AV/AC primer and amplicon size of 520 bp with Deng primer. The DNA isolated from pollen grains of BYVMV infected plant could also produce amplicons of 575 and 520 bp with AV/AC and Deng primer respectively, thus confirmed the presence of virus in the pollen for the first time.

Experiment on *in vitro* co-cultivation of *P. indica* with okra seedlings var. Salkeerthi was done. *P. indica* was maintained in PDA medium and *in-vitro* co-cultivation of *P. indica* with okra was done in modified PNM medium. Microscopic examination of roots revealed presence of mycelium in root surface after ten days of co-cultivation. Colonisation inside roots was observed after 20 days of co- cultivation, but mature chlamydospores in chains were observed within the root cells after 25 days of co-cultivation.

In vivo co-cultivation of *P. indica* with okra var. Salkeerthi in potting mixture mixed with 1 per cent mycelium of *P.indica* revealed that chlamydospores were seen inside the root cells after 25 days of co-cultivation. *P. indica* colonisation enhanced growth of okra seedlings under *in vivo* conditions.

P. indica-colonisation resulted in increase in root and shoot biomass and production of more number of secondary roots under *in vitro* conditions. The colonised plants showed 29 and 50 per cent increase in the shoot and root biomass respectively over the un-colonised plants after 20 days of co-cultivation. Root length was significantly higher in *P. indica*-colonised plants (average root length of 12.6 cm) compared to the un-colonised plants (8.48 cm) after 20 days of co- cultivation. Colonisation of *P. indica* resulted in 49 per cent increase in secondary roots formation in bhendi compared to the control plants.

Evaluation of *P. indica*-priming against the natural incidence of BYVMV and artificial inoculation by grafting was done and the results revealed that *P. indica*-priming reduced the D. I. by 57 per cent and V. I. by 56 per cent over control plants after 40 days of sowing; but D. I. and V. I. was only 40 percent and 53 per cent respectively after 55 days of sowing. Evaluation of *P. indica*-primed okra seedlings and plants against artificial inoculation of BYVMV by grafting revealed that pre- and post-inoculation of the fungus in artificially infected okra plants also reduced the V. I. by 56 per cent and 24 per cent respectively after 30 days of grafting.

Growth parameters were significantly higher in *P. indica*-primed plants under natural incidence of BYVMV than in the control plants except on the number of leaves. Average plant height was significantly higher in *P. indica* colonised plants (43.72) than in un-colonised plants (36.28 cm). *P. indica*-primed plants exhibited

larger leaf area (208.09 cm^2) compared to the non-primed plants (167.14 cm^2). There was 48 and 62 per cent increase in root and shoot fresh weight respectively in *P.indica*-colonised plants over control. Accordingly, root and shoot dry weight was also higher in *P.indica*-colonised plants.

PCR analysis of naturally infected okra plants revealed that virus titre was less in *P. indica*-colonised plants compared to the non-colonised diseased plants.

Biochemical analysis of *P.indica*-primed and non-primed plants under natural incidence of BYVMV and artificial inoculation by graft transmission of virus was done after 40 DAS and 30 DAG respectively. There was increase in chlorophyll and total soluble proteins, and high activities of antioxidant enzymes in *P. indica*-colonised plants. Infection of okra with BYVMV led to a significant reduction in chlorophyll content in particular chlorophyll b content. *P. indica* priming in infected plants significantly enhanced the chlorophyll content; there was a 3-fold increase in total chlorophyll and 3.2-fold increase in chlorophyll b content under natural incidence of BYVMV compared to diseased control. In pre-inoculation of *P. indica* followed by graft transmission of the virus, total chlorophyll content was increased by 176 per cent over diseased control. Total soluble protein content was significantly higher, a 121 per cent and 147 per cent increase was noticed in *P. indica*-primed plants under natural incidence of BYVMV and in pre-inoculation of *P. indica* followed by grafting.

Oxidative and scavenging enzyme activities were found to be higher in *P. indica*-primed plants. Under natural incidence of BYVMV, *P. indica*-primed plants showed 94 per cent and 226 per cent increase in peroxidase (PO) and polyphenol oxidase (PPO) activity respectively. There was 48 per cent increase in activities of both catalase and ascorbic acid oxidase in *P. indica*-primed plants over the diseased control under natural incidence. *P. indica* pre-primed plants artificially challenged with BYVMV recorded maximum PO, PPO, catalase and ascorbic acid oxidase activities which were increased by 109, 176, 96 and 56 per cent respectively over the diseased control. Phosphatase activity was drastically decreased in the virus infected plants but *P. indica*-priming maintained phosphatase activity in diseased plants. The biochemical activities were significantly increased in post-inoculation of the fungus,

but were not substantially high as in the pre-inoculation followed by graft transmission of the virus.

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Appendix

Appendix - I

Potato Dextrose Agar (PDA) medium

Potato: 200 g

Dextrose: 20 g

Agar: 20 g

Distilled water: 1 L

Appendix - II

Plant Nutrient Medium (PNM)

5 mM KNO_3 - 0.5 g

2 mM MgSO4. $7H_2O$)- 0.48 g

2 mM $Ca(NO3)_2$ - 0.472 g

Fe - EDTA - 2.5 ml

Micronutrient mix - 1 ml

Agar - 10 g

Distilled water - 1 L

After sterilization, pH of media was adjusted by adding 2.5 ml filter sterilized 1 M KH_2PO_4.

Micronutrient mix composition

70 mM H_3BO_3

14 mM $MnCl_2.4H_2O$

0.5 mM $CuSO_4.5H_2O$

1 mM $ZnSO_4.7H_2O$

0.2 mM $Na_2MoO_4.2H_2O$

10 mM NaCl

0.01 mM $CoCl_2. 6H_2O$

Fe - EDTA

2.5 g $FeSO_4.7H_2O$ in 400 ml distilled water, add 3.36 g Na_2EDTA, boil the solution for 30 min and make up final volume to 450 ml.

Appendix - III

Estimation of Protein

1. **0.1 M Sodium acetate buffer (pH 4.7)**

   Stock solutions

   A: 0.2 M solutions of Acetic Acid (11.5 ml in 1000 ml)

   B: 0.2 M solutions of Sodium acetate (16.4 g in 1000 ml)

   22.7 ml of A mixed with 27 ml of B, male into 100 ml

2. **Preparation of stock dye solution for estimation of protein**

   100 mg of Coomassie brilliant blue G-250 dissolved in 50 ml of 95 % ethanol and 100 ml of 85 % Orthophosphoric acid was added. The volume was made up to 1 litre with water and kept at 4°C.

Appendix - IV

Buffers for Enzyme analysis

1. **0.1 M Sodium phosphate buffer (pH 6.5)**

   Stock solutions

   A: 0.2 M solutions of monobasic sodium phosphate (27.8 g in 1litre)

   B: 0.2 M solutions of dibasic sodium phosphate (53.65 g in 1 litre)

   68.5 ml of A mixed with 31.5 ml of B diluted to a total of 200 ml

2. **0.067 M Phosphate buffer (pH 7)**

   Dissolve 3.522 g KH_2PO_4 and 7.298 g $Na_2HPO_4.2H_2O$ in distilled water and make up to 1000 ml.

3. **50 mM Sodium Citrate buffer (pH 5.3)**

   Stock solutions

   A: 0.1 M solutions of citric acid (21.01 g in 1000 ml)

   B: 0.1 M solutions of sodium citrate (29.41 g in 1000 ml)

   16 ml of A is mixed with 34 ml of B, diluted to a total of 100 ml

4. **Substrate Solution (Phosphatase assay)**

   Dissolve 1.49 g EDTA, 0.84 g citric acid, and 0.03 g *p*-nitro phenyl phosphate in 100 ml water and adjust pH to 5.3

Abstract

Management of *Bhendi yellow vein mosaic virus* using beneficial fungal root endophyte *Piriformospora indica*

by

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Abstract of the thesis
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ABSTRACT

Management of *Bhendi yellow vein mosaic virus* using beneficial fungal root endophyte *Piriformospora indica*

The study entitled 'Management of *Bhendi yellow vein mosaic virus* using beneficial fungal root endophyte *Piriformospora indica*' was conducted at College of Agriculture, Vellayani during 2018 - 2020 with the objective to evaluate beneficial fungal root endophyte, *P. indica* for the management of *Bhendi yellow vein mosaic virus* (BYVMV) and to elucidate the role of antioxidants in the tripartite interaction.

BYVMV infected okra samples were collected from okra fields of 3 different locations of Thiruvananthapuram district *viz.*, Vellayani, Pappanchani and Palappur. All the surveyed fields were severely infected and the highest disease incidence (D. I.) of 100% and vulnerability index (V. I.) of 71.07 was recorded in okra var. Kiran in Palappur. Characteristic symptoms observed in the surveyed locations include vein clearing, vein thickening, reduction in leaf size, stunted growth and reduced fruit set, fruits if formed were bleached, hardened and deformed. Vein clearing on flower buds was also observed.

Total DNA of infected plant samples were isolated using Qiagen's DNeasy Plant DNA extraction Mini Kit. The quantity and quality of DNA was assessed. The presence of the virus in the diseased samples were confirmed at molecular level by PCR using Deng and AV/AC primers specific to coat protein (CP) of BYVMV (*Begomovirus*) that could produce amplicons of 500 bp and 550 bp respectively in all infected samples. The pollen collected from the infected plants also produced amplicons confirming the presence of the virus.

In vitro co-cultivation studies of *P. indica* in okra seedlings var. Salkeerthi in PNM medium revealed the fungal colonisation and formation of chlamydospores 25 days after co-cultivation. *P. indica*-colonisation enhanced shoot and root biomass; and produced more number of secondary roots.

A pot culture experiment was conducted to evaluate the effect of *P. indica*-priming okra var. Salkeerthi under natural incidence of BYVMV. *P. indica*-priming

significantly reduced the D. I. by 57 and V. I. by 56 per cent over control and also enhanced plant growth with increased root and shoot biomass, leaf area and plant height. There was 48 and 62 per cent increase in root and shoot fresh weight respectively in *P. indica*-colonised plants over control. Biochemical analysis of *P. indica*-primed and non-primed plants under natural incidence of BYVMV revealed increase in chlorophyll and total soluble proteins, and high activities of antioxidant enzymes in *P. indica* colonised plants. Total chlorophyll content was highest (1.32 mg g^{-1} fw) in *P. indica*-primed plants and least in BYVMV infected plants (0.20 mg g^{-1} fw). There was a 3-fold increase in total chlorophyll content in *P. indica*-primed plants under natural incidence compared to the diseased control. Chlorophyll b content was drastically reduced in diseased plants but *P. indica*-priming resulted in 3.2-fold increase in chlorophyll b. Total soluble protein content was increased by 121 per cent in *P. indica*-primed plants. Peroxidase (PO) and polyphenol oxidase (PPO) activities were increased by 94 per cent and 226 per cent respectively. There was 48 per cent increase in activities of both catalase and ascorbic acid oxidase in *P. indica* -primed plants over the diseased control. The decreased disease incidence and severity are attributed to the increased activities of antioxidant enzymes viz., peroxidase, polyphenol-oxidase, catalase and ascorbic acid oxidase.

Effect of *P. indica*-priming (pre- and post- inoculation) on artificial inoculation of the virus by grafting was also evaluated. Both pre- and post-inoculation of the fungus in artificially infected okra plants reduced the V. I. by 56 and 24 per cent respectively. Similar trend as in case of natural incidence was also observed in biochemical activities in *P. indica*-primed okra plants under artificial inoculation. In pre-inoculation of *P. indica* followed by graft transmission of the virus, total chlorophyll and soluble protein contents were increased by 176 and 147 per cent respectively; PO and PPO activities by 109 and 176 per cent respectively; and catalase and ascorbic acid oxidase by 96 and 56 per cent respectively over the diseased control. Phosphatase activity was drastically decreased in the virus infected plants. The biochemical activities were significantly increased in post-inoculation of the fungus, but were not substantially as in the pre-inoculation followed by grafting of the virus.

Thus, the present study revealed that *P. indica* could be exploited for the management of BYVMV on a prophylactic basis. Remission of the symptom in *P. indica*-colonised okra plants was attributed to the enhanced chlorophyll, soluble proteins, PO, PPO, catalase and ascorbic acid oxidase. Further studies should be conducted to elucidate the role of other antioxidants, and molecular mechanisms involved in this host-endophyte-virus interactions. Field and multi-locational studies should also be conducted for confirmation of the results.

സംഗ്രഹം

"*പിരിഫോർമോസ്പോറ ഇൻഡിക്ക* എന്ന വേരിൽ അന്തർവ്യാപനശേഷിയുള്ള മിത്ര കുമിൾ ഉപയോഗിച്ചു വെണ്ടയിലെ മഞ്ഞ ഞരമ്പു മൊസൈക് വൈറസിന്റെ നിയന്ത്രണം" എന്ന വിഷയത്തെ ആസ്പദമാക്കി വെള്ളായണി കാർഷിക കോളേജിലെ സസ്യരോഗ വിഭാഗത്തിൽ 2018 – 2020 കാലയളവിൽ പഠനം നടത്തുകയുണ്ടായി.

പഠനത്തിന്റെ ഭാഗമായി തിരുവനന്തപുരം ജില്ലയിലെ മൂന്നു വ്യത്യസ്ത വെണ്ട കൃഷി ചെയ്യുന്നിടങ്ങളിൽ നിന്നും മൊസൈക് രോഗം ബാധിച്ച സസ്യ ഭാഗങ്ങൾ ശേഖരിച്ചു. വൈറസ് രോഗവ്യാപനവും രോഗതീവ്രതയും വിലയിരുത്തി. ഏറ്റവും ഉയർന്ന രോഗബാധയും (100 ശതമാനം) തീവ്രതയും (71.07) പാലപ്പൂര് മേഖലയിൽ കൃഷി ചെയ്ത കിരൺ എന്ന ഇനത്തിലാണ് കണ്ടെത്തിയത്. ഇല ഞരമ്പുകൾ മഞ്ഞളിച്ചു തെളിഞ്ഞുകാണുക, പുതിയതായി ഉണ്ടാകുന്ന ഇലകൾ മഞ്ഞളിച്ചു കുറുകി വലുപ്പം കുറയുക, കായ്കൾ ഉണ്ടായാൽ അവയുടെ വലുപ്പം കുറഞ്ഞു മഞ്ഞളിച്ചു കാഠിന്യമുള്ളതായി തീരുക, ക്രമേണ ചെടിയുടെ വളർച്ച മുരടിച്ചു ചെടി നശിച്ചുപോകുക എന്നിവയായിരുന്നു പ്രധാന രോഗലക്ഷണങ്ങൾ. രോഗം ബാധിച്ച സസ്യ ഭാഗങ്ങളിൽ നിന്ന് ഡി. എൻ. എ. വേർതിരിച്ചെടുക്കുകയും, ഡെങ്, എവി/എസി എന്നീ പ്രൈമറുകൾ ഉപയോഗിച്ച് പി. സി. ആർ. ടെസ്റ്റിന് വിധേയമാക്കുകയും രോഗഹേതുവായ വൈറസിന്റെ (വെണ്ടയിലെ മഞ്ഞ ഞരമ്പു മൊസൈക് വൈറസ്) സാന്നിധ്യം സ്ഥിരീകരിക്കുകയും ചെയ്തു.

വെണ്ട തൈകളുടെ വേരിൽ *പി. ഇൻഡിക്ക*യുടെ അന്തർവ്യാപന ശേഷിയും വളർച്ചയും വിലയിരുത്തി. *പി. ഇൻഡിക്ക* കോളണൈസേഷൻ ചെടിയുടെ വളർച്ചയെ ത്വരിതപ്പെടുത്തുകയും ശാഖാ വേരുകളുടെ ഉത്പാദനം വർദ്ധിപ്പിക്കുകയും ചെയ്തു. കൂടാതെ ഇലകളുടെ എണ്ണവും വലുപ്പവും കൂട്ടുകയും ചെയ്തു. *പി. ഇൻഡിക്ക* പ്രൈമിംഗ് വെണ്ടയിലെ മഞ്ഞ ഞരമ്പു മൊസൈക് രോഗത്തിന്റെ സ്വാഭാവിക വ്യാപനവും തീവ്രതയും യഥാക്രമം

ശതമാനവും, 56 ശതമാനവും ആയി കുറച്ചു. ബയോക്കെമിക്കൽ പഠനം നടത്തിയതിൽ നിന്നും പ്രൈം ചെയ്യാത്ത വൈറസ് രോഗം ബാധിച്ച ചെടികളെക്കാൾ ഹരിതകം 3 മടങ്ങും, പ്രോട്ടീൻ 121 ശതമാനവും *പി. ഇൻഡിക്ക* പ്രൈം ചെയ്ത ചെടികളിൽ വർദ്ധിക്കുന്നതായി കാണപ്പെട്ടു. നിരോക്സീകരണ എൻസൈമുകളായ പെറോക്സിഡേസ്, പോളിഫിനോൾ ഓക്സിഡേസ്, കാറ്റലേസ് എന്നിവയുടെ പ്രവർത്തനം യഥാക്രമം 94, 226, 48 ശതമാനം എന്നീ തോതിൽ *പി. ഇൻഡിക്ക* പ്രൈം ചെയ്ത ചെടികളിൽ വർദ്ധിച്ചു.

പി. ഇൻഡിക്ക പ്രൈമിംഗ് വെണ്ടയിലെ ഗ്രാഫറ്റിംഗ് വഴിയുള്ള കൃത്രിമ രോഗ വ്യാപനത്തെ എത്രത്തോളവും എങ്ങനെയും തടയുന്നു എന്നതിനെക്കുറിച്ചും പഠനം നടത്തി. കൃത്രിമ മൊസൈക് രോഗ ബാധയ്ക്ക് മുൻപും ശേഷവുമുള്ള *പി. ഇൻഡിക്ക* പ്രൈമിംഗ് രോഗതീവ്രത യഥാക്രമം 56 ശതമാനവും, 24 ശതമാനവും ആയി കുറച്ചു. എന്നാൽ മുൻകൂട്ടിയുള്ള *പി. ഇൻഡിക്ക* പ്രൈമിംഗ് ആണ് മൊസൈക് രോഗത്തെ ഫലപ്രദമായി നിയന്ത്രിക്കുന്നതെന്നു കണ്ടെത്തി. *പി. ഇൻഡിക്ക* പ്രൈം ചെയ്യാത്തതും രോഗം ബാധിച്ചതുമായ ചെടികളെക്കാൾ ഹരിതകം, പ്രോട്ടീൻ, നിരോക്സീകരണ എൻസൈമുകളായ പെറോക്സിഡേസ്, പോളിഫിനോൾ ഓക്സിഡേസ്, കാറ്റലേസ് എന്നിവയുടെ പ്രവർത്തനവും *പി. ഇൻഡിക്ക* പ്രൈം ചെയ്ത വെണ്ട ചെടികളിൽ വർദ്ധിക്കുന്നതായി കാണപ്പെട്ടു.

ചെടികളുടെ വളർച്ചയ്ക്ക് അത്യാവശ്യമായ ഫോസ്ഫറ്റേസ് എൻസൈമിന്റെ പ്രവർത്തനം രോഗം ബാധിച്ച ചെടികളിൽ ഗണ്യമായി കുറയുകയുണ്ടായി. എന്നാൽ *പി. ഇൻഡിക്ക* പ്രൈം ചെയ്ത ചെടികളിൽ ഈ എൻസൈമിന്റെ പ്രവർത്തനം സാധാരണ തോതിൽ നിലനിൽക്കുന്നതായി കാണപ്പെട്ടു.

വേരിൽ അന്തർവ്യാപനശേഷിയുള്ള മിത്ര കുമിളായ *പി. ഇൻഡിക്ക* വെണ്ടയുടെ വളർച്ച കൂട്ടുന്നതോടൊപ്പം മാരകമായ മഞ്ഞ ഞരമ്പു വൈറസ് രോഗത്തെ ഫലപ്രദമായി നിയന്ത്രിക്കുമെന്നും കണ്ടെത്തി.