Management of *Blackeye cowpea mosaic virus* using natural products from botanicals and the fungal root endophyte *Piriformospora indica*

by

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(2017-11-065)

THESIS

Submitted in partial fulfilment of the

requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT PATHOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695522

KERALA, INDIA

2019

DECLARATION

I, hereby declare that this thesis entitled **"Management of *Blackeye cowpea mosaic virus* using natural products from botanicals and the 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.

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CERTIFICATE

Certified that this thesis entitled **"Management of *Blackeye cowpea mosaic virus* using natural products from botanicals and the fungal root endophyte *Piriformospora indica*"** is a record of research work done independently by Mr. Chandran K. 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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ACKNOWLEDGEMENT

It is my privilege to record my deep sense of gratitude and indebtedness to ***Dr. Joy M.*** *Associate professor and Head, Department of Plant Pathology and Chairperson of my Advisory Committee for his expert guidance, constant encouragement, moral support and selfless help in the successful completion of this research programme.*

I am immensely grateful to ***Dr. Radhika N.S,*** *Assistant Professor, Department of Plant Pathology for her inspiring guidance, encouragement, care and ever- readiness to help me during all stages of the study.*

My heartfelt thanks to ***Dr. Ambily Paul.,*** *Assistant Professor (Agrl. Entomology), AINP on Pesticide Residue for her support, valuable suggestions, keen interest, technical advices and incessant motivation throughout the research work.*

I express my profound gratitude to ***Dr. Sarada S,*** *Assistant Professor (Horticulture), Department of Vegetable Science for her generous timely help, unstinting support, suggestions and passionate approach rendered during the period of research work.*

I wish to express my sincere thanks to ***Dr. K. Umamaheswaran,*** *Professor, Department of Plant Pathology for his valuable guidance, help, encouragement and suggestions rendered throughout the study period.*

My special thanks to ***Dr. Susha S. Thara, Dr. Heeera G, Dr. Sreeja S.V, Dr. Ayisha*** *and* ***Dr. Pramod,*** *Assistant Professors, Department of Plant Pathology for their ever willing help and moral support.*

I owe my deepest gratitude to Dr. Nisha, Assistant Professor (Agrl Entomology), Department of Nematology and Dr. Kiran A. G. (Assistant professor in contract), Department of Plant Biotechnology for their timely help, guidance and support.

I gratefully acknowledge the utmost help and encouragement of Reshma chechi, Seethala chechi, Aparna chechi and Elizabeth for their timely technical help and co-operation rendered throughout study period.

I am greatly obliged to non-teaching staff of Department of Plant Pathology Saritha chechi and Jaykumar chettan for their help and co-operation rendered throughout the course work.

I also express my gratitude towards my lovable seniors Safeer, Ananthu, Anjana, Agnes, Madhu, Nisha, Rahila, Amritha chechi, Elizabeth chechi, and Chinnu chechi for their support, timely advice and help during the study period. I extend my thanks to juniors Haritha, Deena, Divya, Veni, Athira, Anitt, Theju, Aswathi, Chippy and Arya for their support and friendship.

I am filled with emotions of gratitude for the sincere assistance and friendly ambience given by my dearest companions Bincy, Safana, Shilpa, Bhavana, Athira, Jyothi, Deepthi, Deepa and Pavan throughout my PG life.

It is my pleasure to express my special thanks to Suresh, Manikandan, Priyanka, bency and chippy chechi for their help and support during my PG life.

I am thankful to Kerala Agricultural University for the technical and financial assistance for carrying out my research work. I am also grateful to Advanced Research Centre for Plant Disease Diagnosis (ARCPDD) and Department of Plant Pathology, College of Agriculture, Vellayani, for the technical support.

My heartful love and gratitude to my amma, appa, thambi and akka for their moral support.

Chandran K

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Per cent
µg	Microgram
µL	Microlitre
µm	Micrometer
^{0}C	Degree Celsius
AMF	Arbuscular mycorrhizal fungi
AVPs	Antiviral principles
BlCMV	*Blackeye cowpea mosaic virus*
CABMV	*Cowpea aphid-borne mosaic virus*
CD	Critical difference
CRD	Completely Randomized Design
cm	centimeter
DAI	Days after inoculation
DAC-ELISA	Direct antigen coating-Enzyme linked immunosorbent assay
DIBA	Dot immunobinding assay
DAC	Days after co-cultivation
EDTA	Ethylene diamine t
et al	And other co workers
g	Gram
h	Hour
KM	Kafer medium
kDa	Kilo Dalton
L	Litre
MS medium	Murashige and Skoog medium
M	Molar
mM	Milli molar
ml	millilitre
mg	milligram
mm	Milli meter

min.	Minute
nm	Nano meter
NCM	Nitro cellulose membrane
OD	Optical density
Pi-WDE	*Piriformospora indica*-Water diffusible exudate
PDA	Potato dextrose agar
PVY	*Potato virus Y*
PMV	*Pumpkin mosaic virus*
PO	Peroxidase
PPO	Polyphenol oxidase
PAL	Phenylalanine ammonialyase
PR-proteins	Pathogenesis related protein
PVP	Poly vinyl pyrolidone
PNM	Plant nutrient medium
rpm	Rotations per minute
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
SDM	Spray dried milk
SE (m) $\pm$	Standard error of mean
TBS	Tris buffer saline
TMV	*Tobacco mosaic virus*
ULCV	*Urdbean leaf curl virus*
WAC	Week after co-cultivation
Viz.,	Namely

Introduction

1. INTRODUCTION

Cowpea is a versatile leguminous crop indigenous to Africa (Andrea *et al.,* 2007) and is cultivated in most of the tropical and sub-tropical countries (Mali and Kulthe, 1980; Bashir *et al.,* 2002; Dhanasekar and Reddy, 2015). It is cultivated as vegetable (green pods), pulse grain, green manure or fodder crop. It contains high quality dietary protein, palatability and also improves soil fertility. Cowpea is considered as an important source of protein to people in developing countries where the availability of nutrients is limited (Pavithra, 2013).

Cowpea is a principal pulse crop cultivated as vegetable in Kerala. Biotic and abiotic stress are the major constraint in the production of pulses. Cowpea is susceptible to many fungal, bacterial and viral diseases. Among the diseases, viral diseases are devastating which may lead to complete crop failure. Cowpea is infected naturally by 140 viruses (Shoyinka *et al.*, 1997) of which 20 are worldwide having RNA genome (Hampton *et al.*, 1997).

The genus *Potyviridae* comprises of several phytopathogenic viruses which cause great damage to cereals, millets, vegetables, fruits, oilseeds, ornamentals, and fodder (Mohamed, 2010). Among the potyvirus affecting cowpea, Blackeye cowpea mosaic strain *Bean common mosaic virus* and *Cowpea aphid-borne mosaic virus* are the major ones (Frison *et al.,* 1990). In Kerala, the major virus infecting yard long beans (vegetable cowpea) is *Blackeye cowpea mosaic virus* (BlCMV) (Krishnapriya, 2015). BlCMV was initially identified in Florida by Anderson (1955). Seeds infected with BlCMV produced 65 to 100 per cent disease incidence in field and drastically reduced the yield (Puttaraju *et al.*, 2000). The incidence of viral disease under natural field condition is 66.60 per cent (Bashir *et al.*, 2002) and the frequency of seed transmission is as high as 30.90 per cent (Zettler and Evans, 1972).

The genome size of BlCMV ranges between 700 to 800 nm and contains tubular and cylindrical inclusion body with sedimentation coefficient of 157 to 159 S

(Albersio *et al.*, 1979). Among the potyvirus, BlCMV is closely related to *Cowpea aphid-borne mosaic virus* (CABMV) and spreads to the healthy plants by means of seeds and aphids or through mechanical transmission.

Fungicides and antibiotics are the easiest and reliable means for the management of phytopathogenic fungi and bacteria but there are no chemicals or substances that could be used as potent viricides for the management of viral diseases. Exploration of natural products is an alternative to find new and safe viricides. Botanicals are gaining importance for management of viral disease (Kumar *et al.*, 2009) since they are non-toxic, eco-friendly and safe. Application of plant extracts act as elicitors to impart resistance in plants against the virus (El-Sawy *et al.*, 2018). Plants such as *Phyllanthus niruri, Boerhavia diffusa, Vitex trifolia* etc. have been reported as virus inhibitors (Veena, 2007).

Plant exudates released into soil, trigger the activation of arbuscular mycorrhizal (AM) fungi for growth and colonization and to develop symbiotic association with plants. The symbiotic benefits to plants include nutrient uptake, enhanced growth and development and tolerance to pathogens and abiotic factors (Smith and Smith, 2011; Tahat and Sijam, 2012). AMF inside the root receive carbon compounds and deliver nutrients required for plant growth (intraradical mycelium) and mycelium outside the root absorb nutrients from soil (extraradical mycelium) (Smith and Smith, 2011).

Piriformospora indica is a beneficial root colonizing basidiomycetous fungi isolated by Verma in 1998 from xerophytic plants of Thar desert in India. It colonizes plants of both monocots and dicots. The mycelium is white or hyaline, thin walled with dolipore septum, irregularly branched and often shows anastomosis. Septation is irregular, multinucleate and the mature mycelium produces chlamydospores which appears single or in cluster. Spore cytoplasm is densely packed and contains eight to 15 nuclei (Varma *et al.*, 2012). *P. indica* is used as plant growth promoter, biofertilizer, bioregulator, bioherbicide, immune- modulator and phytoremediator

(Oelmuller *et al.,* 2009; Johnson *et al.* 2014; Shrivastava and Varma 2014). Once colonized within the plants, the host plant receives long term benefits from the fungi (Sun *et al*., 2010; Matsuo *et al*., 2015).

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

1. To study the symptomatology and the serological identification of virus.
2. To screen potential botanicals having anti-viral principles for the management of BlCMV in vegetable cowpea and partial characterization of effective botanicals.
3. To evaluate *P. indica* and *P. indica*-water diffusible exudates for the management of BlCMV in vegetable cowpea.
4. To study of mechanism of tolerance / resistance by biochemical changes in the treated plants.

Review of Literature

2. REVIEW OF LITERATURE

Cowpea is an important legume crop cultivated in different parts of the world. It has high nutritive value and is called as "poor man's meat" (Pavithra *et al.*, 2015). It is a staple food for the millions of people and is comparatively tolerant to drought condition but susceptible to diseases caused by pathogens especially to viral diseases.

2.1. MAINTENANCE OF VIRUS AND *Piriformospora indica*

2.1.1. *Blackeye cowpea mosaic virus*

Thomas (1937) reported the mosaic disease of *Vigna unguiculata* in India. *Blackeye cowpea mosaic virus* (BlCMV) was first reported in Florida (Anderson, 1955). There are several reports indicating the wide spread nature of BlCMV in India. Virus isolated from Kenya, Nigeria, Tanzania, Morocco and US which were identified earlier as *Cowpea aphid-borne mosaic virus* are now recognized as BlCMV (Bock, 1973; Patel and Kuwaite, 1982, Dijkstra *et al.*, 1987). Puttaraju *et al.* (2000) reported the incidence of BlCMV in Karnataka and its seed borne nature of the virus was studied by Shankar *et al.* (2009). Krishnapriya (2015) confirmed the virus causing mosaic disease in cowpea in Kerala as BlCMV.

2.1.2. Maintenance of the virus

BlCMV was mechanically transmitted into cowpea and *Chenopodium amaranticolor* using chilled 0.1 M Tris buffer with carborundum powder as abrasive material (Mali and Kulthe, 1980). Givord (1981) used 0.01 M phosphate buffer for transmission of *Bean common mosaic virus* in cowpea and symptoms were similar to that of natural incidence. Symptom was developed in most of the mechanically inoculated yard-long bean using phosphate buffer for sap extraction (Wall *et al.*, 1996). Radhika (1999) found that phosphate buffer was effective in mechanical transmission of virus into cowpea and *C. amaranticolor*.

Louis (2003) recorded 90 per cent transmission of *Pumpkin mosaic virus* in pumpkin by using 0.1 M potassium phosphate buffer as extraction buffer for sap transmission. Kumar and Aswathi (2007) used distilled water for extracting *Bitter*

gourd mosaic virus for sap transmission. Kareem and Taiwo (2007) used infected leaf with di-potassium hydrogen orthophosphate buffer (1:2) for mechanical inoculation of virus into cowpea plants. Potassium phosphate buffer was used for the transmission of *Urdbean leaf curl virus* into primary leaf of blackgram (Karthikeyan *et al.*, 2009).

Madhusudhan *et al.* (2011) used 100 mM phosphate buffer for transmission of *Tomato mosaic virus* and *Tobacco mosaic virus* in tomato. 0.1 M Tris buffer with 0.005 M EDTA trisodium salt was used by Dhanasekar and Reddy (2015) to transfer the CABMV in cowpea plants and recorded 100 per cent transmission efficiency. The transmission range of 88 to 96 per cent was achieved when potassium phosphate buffer of 0.05 M was used for transmission of BlCMV in cowpea (Pavithra *et al.*, 2015). Virus was transmitted into cowpea and *C. amaranticolor* using sodium phosphate buffer (Alex, 2017).

2.1.3 Symptomatology

The type of symptoms produced may vary depending on the viral strains and host species (Anderson, 1955). Albersio *et al.* (1979) studied the symptomatology of BlCMV in artificially inoculated host plants. The symptoms observed were mosaic pattern in *Crotalaria spectabilis* and *Macroptiliumatro purpureum*; mottling in *Glycine max* and *Nicotiana benthamiana*; local lesions in *Ocimum basilicum* and *C. amarnaticolor* and epinasty, necrosis and yellowing of leaves in *Phaseolus vulgaris*.

Mali and Kulthe (1980) studied the seed borne nature of potyvirus and the symptoms produced by the virus in cowpea. The symptoms include mild and irregular mosaic followed by yellow mottling, puckering, slight distortion and arching of trifoliate leaves. Givord (1981) observed that BlCMV inoculated cowpea plants produced symptoms like leaf chlorosis and vein clearing at seven days after inoculation (DAI); growth reduction, chlorosis and dark green blotches in leaves at 13 DAI; and vein banding, mosaic, leaf distortion, puckering of leaves and stunting of plants at 16 DAI.

BlCMV produced symptoms *viz.,* necrotic local lesion, general stunting, mosaic with vein clearing and green vein banding in cowpea (Collins *et al.*, 1985). According to Sreelakha (1987) and Ouattara and Chambliss (1991), mechanically inoculated cowpea plants with BlCMV produced symptom within 14 days of inoculation. The symptoms include chlorosis, mild vein clearing, dark and light green patches, mosaic mottling, vein banding, interveinal chlorosis and stunting. The affected plants rarely produced flower and pods.

Saric (1991) studied the symptomatology and host range of BlCMV by mechanical inoculation into different plants. The symptoms produced on host plants were chlorosis, yellow mosaic and vein banding in trifoliate leaves of cowpea. The virus produced systemic infection in *Phaseolus vulgaris, Glycine max, Pisium sativum* and *Nicotiana clevelandii* whereas, localized symptoms were observed in *Phaseolus lunatus, Gomphrena glohosa, Chenopodium amaranticolor* and *C. quinoa.* Plants like *Vica faba, Trifolium pretense, Ocimum basilicum* and *N. tabacum* were free of infection. Radhika (1999) observed yellow chlorotic local lesions in BlCMV inoculated *C. amaranticolor* leaf. The virus infected cowpea seedling produced specific symptoms like downward curling with mosaic or vein clearing in primary cotyledonary leaves followed by mosaic, vein banding and leaf malformation in successive leaves. The infected plants were stunted with bleached and malformed leaves (Hao *et al.*, 2003). Shilpashree (2006) studied the symptom produced by BlCMV on inoculated cowpea plants as green banding, mosaic mottling and leaf distortion whereas, the surveyed plants exhibited systemic mosaic with dark green and light patches, distortion and cupping of leaves. BlCMV produced mosaic, vein banding, leaf distortion, reduction in leaf size and flower malformation (Krishnapriya, 2015). Dhanasekaran and Reddy (2015) reported that artificially inoculated cowpea plants with BlCMV produced mosaic, vein banding, vein clearing, upward and downward leaf curling, interveinal chlorosis, crinkling, mottling and chlorotic patches in leaves. According to Alex (2017), BlCMV virus inoculated on cowpea plants produced vein clearing on trifoliate leaves followed by mottling, mosaic, vein banding and leaf distortion and

the infected plants become stunted whereas, the virus produced local lesions in *C. amaranticolor*.

2.1.4. *Piriformospora indica*

P.indica is a beneficial endophytic root colonizing sebanicales fungus with multifunctional activities *viz.,* increased seed production, nutrient uptake, biomass and (a)biotic stress tolerance in plants (Johnson *et al.*, 2014 and Gill *et al.*, 2016). It colonizes plants which are having forestry, agriculture and horticultural importance (Shrivastava and Varma, 2014; Uma *et al.*, 2017).

2.1.5. Maintenance of fungal endophytes

Endophytes require specific media for growth and development. *P. indica* fungal culture was maintained in complete agar medium at optimum temperature for growth and spore production (Serfling *et al.*, 2007). Sun and Oelmuller (2010) observed enhanced growth when *P. indica* was cultured in modified Kafer medium and maintained at 25°C with 12 h photoperiod. *P. indica* inoculated on Potato Dextrose Agar (PDA) medium produced hyaline, white mycelium and chlamydospores at 15 DAI (Sun *et al.*, 2010). Active stage of fungus was maintained by regular sub-culturing at 10 days interval in PDA (Satheesan *et al.*, 2012). Kumar *et al.* (2012) observed increased growth of mycelium and early spore production in Kafer medium amended with soluble starch. Johnson *et al.* (2013) optimized the nutrient requirements of *P. indica* by modifying the Kafer medium and maintained the culture by transferring four weeks old culture into fresh medium and incubated in dark at 22-24°C. Hill and Kafer medium supported the maximum growth of *P. indica* and was maintained at 30±2 °C (Krishnaveni *et al.*, 2014). Maximum mycelial growth and chlamydospore production were recorded in four per cent jaggery medium, and in medium containing nitrogen, yeast extract and peptone (Varma *et al.*, 2014). Deka and Baishya (2015) maintained the actively growing culture of *P. indica* in PDA broth. Kilam *et al.* (2015) maintained *P. indica* in Hill and Kafer medium with one per cent agar.

P. indica was multiplied and maintained in Kafer medium (KM) which was incubated at 28°C in dark for two weeks (Kim *et al.*, 2017). Nivedita *et al.* (2017)

maintained the *P. indica* culture by inoculating in Aspergillus medium at 30°C in dark conditions. Maximum biomass of *P. indica* was obtained within a week in jaggery medium (Uma *et al.*, 2017). Su *et al.* (2017) maintained *P. indica* in modified Kafer medium at 25°C for 5 days in dark. *P. indica* culture was incubated in Aspergillus medium standardized by Hill and Kafer at room temperature and sub-cultured every two months (Vassilev *et al.*, 2017). The fungal endophyte was maintained in PDA and mycelium attained full growth in nine days at 28°C (Alex, 2017; Jisha *et al.*, 2018; Anith *et al.*, 2018).

2.2. DETECTION OF *Blackeye cowpea mosaic virus* USING SEROLOGICAL METHOD

Detection of pathogen is important for diagnosis of diseases caused by pathogen and its management. For viral diseases, detection methods may be either serological or molecular. In 1960's there was rapid development in serological identification of virus by development of ELISA and DIBA (Martin *et al.*, 2000).

2.2.1. Enzyme Linked Immunosorbent Assay (ELISA)

ELISA was widely used in the rapid detection of viruses present in different plant parts (Mwaipopo *et al.*, 2017). Ouattara and Chambliss (1991) carried out disease resistant breeding of cowpea against *Blackeye cowpea mosaic virus* by transferring disease resistant genotypes from "White Acre-BVR" to susceptible variety "Califorina Blackeye No 5" and the resistance was confirmed by ELISA. Huguenot *et al.* (1993) used different ELISA procedures to distinguish CABMV and BlCMV and finally concluded that CABMV was BlCMV.

Wall *et al.* (1996) conducted Protein A Sandwich ELISA for scoring BlCMV incidence in cowpea cultivars. Hao *et al.* (2003) conducted an experiment to identify virus infected seeds in catjang bean and yard long bean by plate trapped antigen ELISA and sample which showed positive results were further analyzed by triple antibody sandwich ELISA to differentiate BlCMV and CABMV infected samples.

Shankar *et al.* (2009) studied different seed borne virus in cowpea from136 seed lots and 43 lots were positive in ELISA against BlCMV. Cowpea was infected by many virus and some were seed transmitted. Udhayashankar *et al.* (2010) studied the percentage seed transmission of BlCMV and the virus was confirmed serologically by ELISA in the seeds. Madhusudhan *et al.* (2011) conducted an experiment to study the antiviral property of plant extracts and quantified the viral concentration in treated plant using DAC-ELISA.

Pavithra *et al.* (2015) conducted a survey for assessing the severity of BlCMV in southern districts of Karnataka and confirmed the virus by using potyvirus antisera in DAC-ELISA. The results indicated that the disease incidence varied from 1 to 70 per cent due to the weather and susceptibility of variety. DAS-ELISA was conducted on 92 cowpea varieties inoculated with CABMV to screen the resistant genotypes and 13 were identified as highly resistant (Dhanasekar and Reddy, 2015). Krishnapriya (2015) and Alex (2017) performed DAC-ELISA and identified the causal agent of mosaic disease in cowpea in Kerala as BlCMV.

2.2.2. Dot Immunobinding Assay (DIBA)

Dot immunobinding assay was used in rapid detection of plant virus even in latent infection (Hibi and Saito, 1985). Powell (1987) reported that the nitro cellulose membrane assay detected the virus even in very low concentrations than the ELISA. Krishnapriya (2015) and Alex (2017) confirmed the viral disease causing mosaic in cowpea as BlCMV by DIBA.

2.3. STANDARDIZATION OF CO-CULTIVATION METHOD FOR *P. indica*

Deshmukh *et al.* (2006) studied the symbiotic relation between *P. indica* and barley plants. It was observed that fungal hyphae penetrated into the intercellular spaces in sub-epidermal layer and did not colonize adjacent cell until the infected cell are colonized completely. In mature cells it forms net like inter and intra cellular colonization. The hyphae penetrated inside the root of epidermal cells within a week, forms dense mass of intercellular hyphae in second week and spores were seen in third week in wheat (Serfling *et al.*, 2007).

Linum album was grown on Gamborg's B5 media inoculated with five day old *P. indica* broth culture in different concentration for colonization; and one per cent broth culture at 11 DAI had higher colonization in roots and produced large amount of lignin (Baldi *et al.*, 2008). Co-cultivation of *P. indica* with *Bacopa monniera* was done in Hill and Kafer medium and *P. indica* colonized *B. monniera* enhanced plant performance and increased the quantitative and qualitative characters of the medicinal plants (Prasad *et al.*, 2008). Co-cultivation of *Arabidopsis thalaina* and *P. indica* was carried out in modified plant nutrient medium (PNM) and *P. indica*-primed plants performed well under drought condition (Sherameti *et al.*, 2008). Kumar *et al.* (2009) prepared co-cultivation medium with 1 per cent *P. indica* mycelium and 10 per cent Hoagland's solution with sterile soil for colonization of the fungus in maize and achieved 70 per cent colonization in 20 days.

Mycelium of *P. indica* grown in Aspergillus A complex liquid medium was washed with sterile water, homogenized and two grams of mycelium was added to 300 g of sterile substrate for co-cultivation with barley (Achatz *et al.*, 2010). Sun *et al.* (2010a) inoculated 2 weeks old *P. indica* fungal disc into the 5 days old Chinese cabbage in half MS medium for 30 days to get the root colonization. Mycelium of *P. indica* in Hill and Kafer broth was filtered, washed and placed in unsterile potting mixture as sandwich layer model for colonization in *Coleus forskohlii* (Das *et al.*, 2012). Two weeks old *Centella asiatica* from MS media was transferred to media containing 1:1 ratio of MS and PDA for the colonization (Satheesan *et al.*, 2012). Roots collected after 45 days of co-cultivation showed chlamydospores and the mycelium penetrated intercellularly in the roots indicating the colonization of the fungus. Co-cultivation for *P. indica* was standardized by Johnson *et al.* (2013) with *A. thaliana* as model plant. Eight to twelve day old *A. thaliana* seedling was transferred into PNM medium containing one week old *P. indica* for effective colonization.

Sugarcane co-inoculated with *P. indica* culture had more than 90 per cent colonization after four weeks and inoculated plants had higher survival rate and

cane yield in both planted and ratoon crop (Varma *et al.*, 2014). Co-cultivation was carried out in PNM media by adding *P. indica* chlamydospores into *A. thaliana* to study the interaction between them (Vahabi *et al.*, 2014). Infected plants grew healthy with no morphological or physiological changes.

Sorghum, pearl millet and finger millet colonized with *P. indica* by adding inoculum to the sterile soil recorded profuse growth and enhanced grain weight (Krishnaveni *et al.*, 2014). Maximum colonization of 65 per cent was observed in *Aloe vera* roots collected after six weeks of co-cultivation with the fungus in MS medium (Sharma *et al.*, 2014). Maize seeds were placed in sterile soil inoculated with *P. indica* and 55 per cent colonization was observed in roots and the colonized plants had enhanced root and shoot biomass (Krishnaveni *et al.*, 2015). *Stevia rebaudiana* co-cultivated with *P. indica* had 50 per cent colonization in four weeks and the colonization increased to 65 per cent with the addition of *Azotobacter chroococum* (Kilam *et al.*, 2015).

Triterpenoids production was enhanced by co-cultivation of *Lantana camera* with *P. indica* (Kumar *et al.*, 2016). Arabidopsis plant co-cultivated for longer time with *P. indica* flowered earlier than un-inoculated plants (Kim *et al.*, 2017). Rice seedlings dipped in chlamydospores suspension of *P. indica* and planted in sterile soil colonized in 14 days (Nassimi and Taheri, 2017). Rice root colonized by *P. indica* had intracellular hyphal growth followed by penetration of hyphae into free spaces, spreading along the entire root (Nivedita *et al.*, 2017). Colonization of 7-9 per cent was observed within seven days of co-cultivated chickpea plants with *P. indica* and maximum colonization was observed on 30 days after inoculation (Narayan *et al.*, 2017). Co-cultivation of *P. indica* with medicinal plants *viz.*, turmeric and isabgol indicated that the colonized plants enhanced the biochemical property as well as yield (Uma *et al.*, 2017).

Six days old *Brassica napus* seedling in MS medium when transferred into one week old *P. indica* culture in PNM medium could produce pear-shaped chlamydospores in epidermis and cortex of roots, increased root hairs and root

biomass (Su *et al.*, 2017). *P. indica* produced chlamydospores seven days after inoculation in roots of cowpea (Alex, 2017).

Co-cultivation of *P. indica* with black pepper was done in sterile vermiculite medium amended with one percent of mycelium (Anith *et al.*, 2018). *P. indica* developed symbiotic association with rice plants on 4 days after inoculation and the symbiosis increased and the association was maintained till the harvest of the crop (Saddique *et al.*, 2018).

2.4. *P. indica*-WATER DIFFUSIBLE EXUDATES

Filtrate from *P. indica* is equally important as that of the fungus. The filtrate contained carbohydrates, saponins, hormones, proteins and enzymes (Badge *et al.*, 2010). Culture filtrate treated plants recorded enhanced biomass production in different crops like tobacco, neem and sunflower (Badge *et al.*, 2010). Water diffusible exudates of *P. indica* could induce cytosolic calcium elevation and growth promotion in *A. thaliana* and Chinese cabbage (Lee *et al.,* 2011 and Johnson, 2014).

In vitro experiments were carried out in wheat, bengal gram, beans, mustard and broccoli, where the filtrate of *P. indica* at low concentration improved the biomass. Filtrates inhibited seed dormancy, improved seed germination and enhanced the seedling growth of the treated plants (Varma *et al.*, 2012a).

Su *et al.* (2017) reported an increase in biomass of *B. napus* when treated with the crushed mycelium of *P. indica* multiplied in Kafer broth and applied through irrigation water.

2.5. MANAGEMENT OF BICMV USING AVPs, *P. indica* AND ITS EXUDATES

Viral disease could be managed by using resistant varieties and virus free planting materials, rouging, vector control or transgenic plant. Effective method for management of viral disease involves the exploitation of natural resources including botanicals with potential anti-viral principles (AVPs) and beneficial endophytes *viz., P. indica*.

2.5.1. Screening of AVPs

Plants are used since ancient times for the management of plant diseases and some of the plants have viral inhibiting property. The continued search of botanicals guarantees safety, quality products and wide diversity of management of viral diseases. This search of antiviral substances against plant viral disease starts with the identification of pokeweed against tobacco mosaic virus in 1925.

Pre-inoculation of *Bougainvillea sp.* and *Eupatorium odoratum* leaf extracts could effectively manage the cowpea mosaic disease (Sreelakha, 1987). *Basella alba, Clerodendrum inerme, Boerhavia diffusa and Nerium odorum* etc., could inhibit the *Tobacco mosaic virus* (Nagarajan., 1990). Hong Xi-Xu *et al.* (1996) screened 75 medicinal plants against Human immunodeficiency virus (HIV) and forty plants including *Punica granatum* exhibited the antiviral property.

Spraying of root extracts of *Mirabilis jalapa* 24 h before virus inoculation inhibited the potato virus completely (Vivanco *et al.*, 1999). Radhika (1999) found that preventive spray of neem oil reduced the viral disease in cowpea significantly than post-inoculation application. Premanathan *et al.* (2000) screened 69 plants against HIV virus and reported high viral inhibiting property of *Cinnamomum cassia* and *Cardiospermum helicacabum*. Different species of *Bougainvillea* was screened against the *Tobacco mosaic virus* and *Sunhemp rosette virus* on *Nicotiana glutinosa* and *Cyamposis teragonoloba* and found that *Bougainvillea xbuttiana* leaf extract was effective in inhibiting the virus when sprayed within 4 h after virus inoculation (Narwal *et al.*, 2001).

Aswathi *et al.* (2015a) reported that *B. diffusa, Phytolacca americana, Dianthus caryophyllus, M. jalapa, Cuscuta reflexa, Bougainvillea spectabilis*, and *Celosia cristata* exhibited viral inhibition when applied on or one day before or immediately after mechanical inoculation of the virus. Identification of antiviral compounds for management of pumpkin mosaic disease was carried by Louis (2003) using medicinal plants such as *Phyllanthus jraternus*, *B. alba, Glycyrrhiza glabra, Plumbago rosea* and *Thespesia populnea*, where weekly spray of *P. rosea* one per cent was effective in management of the viral disease in pumpkin.

Reddy *et al.* (2006) reported that the plant extracts such as *Datura metel, B. diffusa, M. jalapa, Catharanthus roseus, B. spectabilis* and *Azadirachta indica* could significantly reduce *Urdbean leaf crinkle virus* incidence. Seeds treatment with *B. diffusa* leaf extracts at 0.75 per cent concentration and foliar application of *B. diffusa*, *B. spectabilis, M. jalapa* and *C. inerme* significantly reduced the *Bean common mosaic virus* strain-*Blackeye cowpea mosaic virus* in cowpea (Prasad *et al.*, 2008). Karthikeyan *et al.* (2009) screened 25 plant extracts against *Urdbean leaf crinkle virus* and found that all extracts inhibited the virus but *M. jalapa* and *B. spectabilis* significantly inhibited the virus irrespective of time of application.

Kumar and Aswathi (2007) studied the effect of root extracts of *B. diffusa* against the bottle gourd mosaic disease. Continuous spray of this extracts once in a week or a minimum of five to six sprays suppressed the disease and multiple sprays are required as the extract loses its effectiveness within a short period. Lohani *et al.* (2007) reported the anti-viral property of *B. diffusa* against the TMV on *Nicotiana tobaccum*. The root extracts stimulated host resistance and makes the plant less susceptible to viral infection. Veena (2007) studied the antiviral property of *Phyllanthus niruri* and *B. diffusa* on CABMV in cowpea plants which inhibited the virus even at low concentration of one per cent.

Seed treatment followed by foliar application of botanicals such as *A. indica, M. jalapa* and *Terminalia arjuna* were effective in management of *Cucumber mosaic virus* (Kumar and Aswathi, 2009). They also pointed that increase in number of sprays reduced viral incidence, thus increased growth and yield. Rajinimala *et al.* (2009) studied the management of *Bittergourd yellow mosaic virus* with plant extracts, chemicals and biocontrol agents; and observed low disease incidence in *B. spectabilis* treated plants.

Eucalyptus globulus, A. indica, Allium sativum and *Calotropics procera* inhibited *Cotton leaf curl virus*; whereas *Aloe babadensis* and *Datura stramonium* were least effective (Ali *et al.*, 2010). Mohamed (2010) conducted an experiment to find the efficacy of garlic-cloves and onion stem extracts against *Potato virus Y* in local lesion host, *C. amaranticolor*. Al-Ani *et al.* (2011) conducted an

experiment with three plant extracts *viz.,* frog fruit (*Lippi anodiflora*), Datura (*Datura metel*) and Thuja (*Thuja orientalis*) at different concentrations as tuber dipping and foliar application for the management of *Potato virus Y* in potato. The results revealed that all the extracts were effective in reducing the virus upto a month, and there was no differentiation with different concentrations and application in presence of the virus inoculum and aphid populations. Madhusudhan *et al.* (2011) reported that plant extracts from *B. spectabilis* reduced the *Tobacco mosaic virus* and *Tomato mosaic virus* in tobacco of 62 and 59 per cent respectively compared to other extracts. Aqueous leaf extracts of *A. indica, C. aculeatum* and root extracts of *B. diffusa* minimized the urdbean yellow mosaic disease incidence; and increased number of spraying decreased the disease incidence; thus increased the plant height, pod and yield in urdbean and mungbean (Singh *et al.*, 2011). Thuja extract at 0.6 per cent was more effective in suppression of *Watermelon mosaic virus* in *Citrullus lanatus* than 0.2 and 0.4 per cent (Elbeshehy, 2015). Plants having antiviral compound was screened by Asare-Bediako *et al.* (2014) for effective management of okra leaf curl disease and found that 10 per cent aqueous extracts of *A. indica, A. sativum, Carica papaya* and *Bougainvillea sp.* possessed anti-viral and insecticide properties which reduced whitefly population and increased fruit yield.

M. jalapa leaf extract applied prior to virus inoculation could inhibit the virus to 50-60 per cent (Waziri *et al.,* 2015). Aswathi and Singh (2015) mentioned the antiviral property of *B. diffusa (*root extract*), D. metel, C. aculeatum, Solanum melongena, Euphorbia hirta, M. jalapa, Phytolacca americana, A. vera, A. indica, T. arjuna, Ipomoea fistulosa, Tinospora cordifolia,* and *Cuscuta reflexa* (filaments extract) and *C. aculeatum* to different viral diseases.

Elsharkawy and El-Sawy (2015) evaluated different plant extracts and found that seed treatment with one per cent *Plectranthus tenuiflorus* and *M. jalapa* were effective in management of *Bean common mosaic virus*. *B. diffusa* root extracts recorded maximum inhibition of virus when treated 24 h prior to the virus inoculation (Aswathi *et al.*, 2016). Five per cent neem leaf extracts was effective

in reducing disease severity of *Okra yellow vein mosaic virus* and reduced the vector population (Chaudhary *et al.*, 2016). Sharma *et al.* (2017) tested the plant extracts by seed treatment followed by six foliar sprays for viral disease management in watermelon. The increased number of spraying root extracts of *B. diffusa* recorded maximum virus inhibition followed by *A. indica* and *C. aculeatum* with enhanced growth and yield of crops. Aruna *et al.* (2017) studied the possibility of reducing scabrood virus in honey bees by feeding plant extract with sugar syrup and noticed that honey bees fed with 0.8 per cent *P. niruri* significantly reduced the disease. Abu-jafar and Huleihel (2017) reported the antiviral activity of *Eucalyptus camaldulensis* against *Herpes simplex virus* (1 and 2) and *Varicella-Zoster Virus*.

P. niruri and *B. diffusa* at one per cent concentration inhibited BlCMV disease incidence in cowpea and *P. indica* colonized cowpea plants have less disease index (Alex, 2017). She also reported preventive spray of botanicals was effective in managing the disease compared to infected plants. Phytoprotein extract from *B. diffusa* and *C. aculeatum* mixed with bio-enhancer reduced the *Cucumber mosaic virus* in cucumber and *Tomato mosaic virus* and *Tomato leaf curl virus* in tomato (Najam *et al.*, 2017). *C. aculetum* and *B. diffusa* phytoprotein extract mixed with L-Lysine monohydro chloride reduced the severity of *Papaya ring spot virus*, delayed the symptom development or produced mild symptom with increased yield (Najam *et al.*, 2017a). Islam *et al.* (2018) reported that plant extracts of *Celosia cristata, Cynanchum komarovii, Sambucus williamsii, Strobilanthes cusia, Hosta plantaginea, Aschers, Bougainvillea xbuttiana, Brucea javanica, Picrasma quassioides, Sambucus williamsii* etc., were having anti-viral property against TMV. Saleem *et al.* (2018) observed that two per cent garlic extract significantly inhibited the *Urdbean leaf crinkle virus* in green gram followed by *Bougainvillea* and onion extracts. Ginger rhizome extracts @ 200 µg ml^{-1} prior to *Tomato yellow leaf curl virus* inoculation had maximum inhibition of the virus compared to horsemint and silica nanoparticles (El-Sawy *et al.*, 2018).

2.5.2. Management of disease using *P. indica* and its water diffusible exudates

P. indica colonized tomato plants suppressed the symptom expressed by *Pepino mosaic virus* and virus concentration was decreased with increase in colonization (Fakhro *et al.*, 2010). Wang *et al.* (2015) observed that the *P. indica* primed tomato plants induced resistance against *Tomato yellow leaf curl virus* in susceptible variety with enhanced plant growth. *P. indica*-primed cowpea plants were less vulnerable to BlCMV and inhibited more than 50 per cent of BlCMV infection (Alex, 2017).

Incidence of *Barley yellow dwaft virus* was lower in endophytes colonized meadow rye grass compared to non-colonized plants (Lehtonen *et al.*, 2006). Cucumber roots colonized with arbuscular mycorrhizal fungus (AMF), *Glomus mosseae* and plant growth promoting fungus, *Fusarium equiseti* significantly reduced *Cucumber mosaic virus* (Elsharkawy *et al.*, 2012). Squash plants colonized with *Beauveria bassiana* were effective against *Zucchini yellow mosaic virus* (Jaber and Salem, 2014). Muvea *et al.* (2018) reported that the endophytic fungus, *Hypocrea lixii* colonized plants inhibited the replications of *Iris yellow spot virus*.

Lee and Ryu (2016) observed that the leaf colonizing bacteria, *Bacillus amyloliquefaciens* protected chilly against mechanically transmitted *Cucumber mosaic virus* and natural incidence of *Broadbean wilt virus* and *Pepper mottle virus* by enhancing salicylic acid and jasmonic acid defense signaling in plants. Leaf colonizing yeast *Pseudozyma churashimaensis* protected chilly plants against *Xanthomonas axonopodis* in addition to *Cucumber mosaic virus, Pepper mottle virus*, *Pepper mild mottle virus* and *Broadbean wilt virus* by induction of PR-proteins (Lee *et al.* 2017). *B. amyloloquefaciens*-primed plants suppressed *Tobacco streak virus* in cotton (Vinodkumar *et al.*, 2018). The endophytes contain ten antimicrobial polypeptides along with pyrrole and deconicacid imparts antiviral property to the endophytic bacteria.

Culture filtrate from *Streptomyces griseorebens* and *S. cavourensis* recorded maximum per cent inhibition of local lesion expressed by *Cucumber mosaic virus* in *C. amaranticolor* (Shafie *et al.*, 2016). Venkatesan *et al.* (2007) reported that

the foliar application of *P. fluorescens* and plant extracts (*M. jalapa* and *D. metel*) reduced the incidence of *Mungbean yellow mosaic virus* in blackgram. Pseudomonas strains CHA0 with chitin sprayed at regular intervals in banana plantations reduced the *Banana bunchy top virus* compared to Pseudomonas strains CHA0 alone which indicated the increased efficiency of chitin enriched biocontrol agents (Kavino *et al.*, 2008).

2.6. PHYSICAL AND CHEMICAL PROPERTY OF AVP'S

2.6.1 Thermostability

The virus inhibiting property of *B. alba* and *C. inerme* was stable even at 90°C (Nagarajan *et al.*, 1990). The virus inhibiting activity of *C. inerme* remain stable upto 80°C but lost its activity when exposed to 90°C (Prasad *et al.*, 1995). The antiviral principles isolated from *Cleosia cristata* remains same when incubated at 90°C for 10 min but lose its property when exposed to 95°C (Balasubrahmanyam *et al.*, 2000). Narwal *et al.* (2001) studied the stability of AVP extracted from *B. xbuttiana* and the AVP was stable upto 70°C for 10 minutes. *Plumbago rosea* extracts was stable and exhibited higher inhibition against *Pumpkin mosaic virus* in pumpkin when exposed to 70°C for roots extracts and 80°C for mature leaf extracts (Louis, 2003).

2.6.2 Photolysis

Vimalanathan *et al.* (2009) found that the effectiveness of plant extracts against human viruses increased when exposed to particular period of light.

2.6.3 Chemical nature

Nagarajan *et al.* (1990) identified that the glycoproteins containing 74 per cent protein and 26 per cent carbohydrates from *B. alba* and *C. inerme* which inhibited *Tobacco mosaic virus*. Plants treated with the protein fraction of *C. aculeatum* extracts could induce systemic resistance against the virus (Verma *et al.*, 1996). Hong Xi-Xu *et al.* (1996) reported the aqueous extracts *Punica granatum* had phenols and tannins. Protein portion of *M. jalapa* root extract inhibited *Potato virus X, Potato virus Y* and *Potato leaf roll virus* in potato

(Vivanco *et al.*, 1999). *B. xbuttiana* AVP was characterized as lysine rich glycoprotein (Narwal *et al.*, 2001).

Antiviral property of *C. aculeatum* was due to two proteins which induced resistance against TMV in tobacco (Balasubranmanyam *et al.*, 2000). Dunic *et al.* (2010) identified the antiviral activity of *Satureja montana* essential oil which was due to the presence of terpenes and phenol which could reduce TMV and *Cucumber mosaic virus* infection in local lesion host *C. amaranticolor*. *B. diffusa* roots containing glycoprotein was extracted and found that the treated plants had less number of virus particles (Aswathi *et al.*, 2016). *Indian citrus ringspot virus* infected budsticks treated with phytoproteins from roots of *B. diffusa* and leaves of *C. acuelatum* were free from virus which was confirmed with ELISA and RT-PCR (Bishnoi *et al.*, 2017).

2.6.4 Molecular size

Antiviral principles from plants belongs to chenopodiales were protein and its molecular weight ranged from 24 to 38 kDa (Smookler, 1971). Verma *et al.* (1979) found that molecular weight of anitiviral proteins from root extracts of *B. diffusa* ranged between 16 to 20 kDa. Prasad *et al.* (1995) analyzed the molecular weight of active principle from *C. inerme* against TMV as 29 kDa and 34 kDa. Balasaraswathy *et al.* (1998) characterized protein from *B. spectabilis* and identified its molecular weight as 28 kDa. Proteins precipitated from *B. xbuttiana* had 24 kDa and 28 kDa in electrophoresis gradient gel whereas in SDS-PAGE, it had 33 kDa and 28 kDa proteins (Narwal *et al.*, 2001).

2.7. BIOCHEMICAL CHANGES OF HOST PATHOGEN INTERACTION

2.7.1. Total soluble protein

AVP extract from *B. xbuttiana* treated plants had reduced protein content but increase in some specific protein content than untreated plants (Narwal *et al.*, 2001). Sorghum leaf extracts sprayed against squash mosaic disease in summer squash produced more amount of protein and amino acids (Singha, 2002). Louis (2003) reported the decrease in protein content on PMV infected and *Plumbago*

rosea extracts applied plants due to the conversion of protein into defense substances which imparted resistance to the plant. *P. niruri* and *B. diffusa* treated plants had higher protein content which decreased after 15 days of application in cowpea plants for managing viral disease (Veena, 2007).

Total soluble protein accumulated more in *B. spectabilis* treated plant against *Bittergourd yellow mosaic virus* (Rajinimala *et al.*, 2009). *Aloe vera* plants colonized with *P. indica* had low content of protein compared to uncolonized plants (Sharma *et al.*, 2014). *Citrullus lanatus* infected with *Watermelon mosaic virus* was treated with thuja extracts and decreased the soluble protein content compared to untreated infected plants (Elbeshehy *et al.,* 2015). *P. indica* colonized sorghum, pearl millet and finger millet produced two to three fold increase in protein content than uncolonized plants (Krishnaveni *et al.*, 2014). *Potato virus Y* infected plant had higher protein content whereas sea algae extract treated virus infected plants had lower protein content (Hanaa, 2017). Spraying of *P. niruri* and *P. indica* colonized plants produced higher protein content than virus inoculated or healthy cowpea plants (Alex, 2017).

2.7.2. Total Phenol

Singha (2002) studied the biochemical changes in sorghum extracts on squash mosaic disease in summer squash and observed higher content of phenol in AVP treated plants than the infected plants. Phenolic content was decreased in *Pumpkin mosaic virus* infected plants and *P. rosea* treated healthy and infected plants (Louis, 2003) which suggested the pumpkin plants containing higher phenol content was oxidized and converted into substances to suppress the disease. TMV treated plants triggered phenol production which declined after three days but *Celosia cristata* treated TMV inoculated plants have no change in first two days but began to increase from third day onwards (Gholizadeh *et al.*, 2004). The result indicated *C. cristata* induced phenol production to inhibit the virus. Phenol content was increased several fold in *P. niruri, B. diffusa* and *Pseudomonas* treated plants for reducing the viral disease incidence in cowpea (Veena, 2007). Phenol content was increased in AVP treated and ULCV infected plants up to

three days after inoculation and started to decline there after (Karthikeyan *et al.*, 2009). *M. jalapa* treated plant enhanced the phenol production whereas *P. fluorescens* reduced the phenol in blackgram against the urdbean yellow mosaic disease (Venkatesan *et al.*, 2007).

Phenol content of *Aloe vera* was enhanced by *P. indica* compared to un-inoculated plants (Sharma *et al.*, 2014). Phenol content was high in healthy and virucides from sea algae treated potato plants against *Potato virus Y* compared to the virus infected plants (Hanaa, 2017). Banana bunchy top affected plants when treated with *Pseudomonas* strains CHA0 with or without chitin, there was an increase in phenol content which was twice in *Pseudomonas* strain treated plant with chitin compared to healthy plants (Kavino *et al.*, 2008). Phenol content increased from 4 day after application to 16 days and then gradually declined in bittergourd treated with *B. spectabilis* against *Bittergourd yellow mosaic virus* (Rajinimala *et al.*, 2009). Phenol content increased in *Stevia rebaudiana* primed *with P. indica* and it further enhanced with addition of *Azotobacter chroococum* (Kilam *et al.*, 2015). Pre application of *P. niruri* to virus inoculation have much higher phenol content than post application but both having higher phenol compared to virus inoculated and healthy plants (Alex, 2017). She also observed that *P. indica* colonized plants produced gradual increase in phenol even after 30 days of inoculation.

2.7.3. Defense Related Enzyme

Peroxidase activity was increased in both PMV infected and *Plumbago rosea* treated plants in pumpkin whereas there was decrease in Phenylalanine ammonia lyase in both the plants (Louis, 2003). Devi *et al.* (2004) observed rapid increase in peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) enzymes in the treated plants than healthy and diseased plants. Gholizadeh *et al.* (2004) studied the activity of PO, PPO and PAL in TMV infected plants treated with *Celosia cristata*. The results revealed that the virus inoculated plants induced systemic resistance by the induction of enzyme but AVP treated plants had no change or slight decline in enzyme activity.

Cowpea plants treated with *P. niruri, B. diffusa* and *P. fluorescens* accumulated more amount of PO, PPO and PAL in the treated cowpea plants (Veena, 2007). Application of *P. fluorescens* and plant extracts (*D. metel* and *M. jalapa*) induced resistance in black gram against *Urdbean yellow mosaic virus* by enhancing the production of PO, PPO and PAL in treated plants than control (Venkatesan *et al*., 2007). Pre-inoculated treatments (*M. jalapa, B. spectabilis* and *Prosopis chinensis*) and ULCV inoculated plants in urdbean produced defense enzymes such as PO, PPO, PAL and isozyme peroxidase on first day and started to decrease on third day after application (Karthikeyan *et al*., 2009). PO, PPO and PAL activities were increased twice in plantains sprayed with *Pseudomonas* strains CHA0 with chitin than the healthy plants followed by increase the enzyme in *P. fluorescens* strain CHA0 treated plants (Kavino *et al*., 2008). The infection of BYMV in bittergourd was reduced due to significant production of PO and PPO in *B. spectabilis*, *Pseudomonas chlororaphis* and *P. fluorescens* treated plants (Rajinimala *et al*., 2009).

Higher PAL enzyme activity was observed in *P. indica* co-cultivated plants in early hours and the activity decreased as the colonization was maintained for longer duration (Baldi *et al*., 2008). Chinese cabbage plants colonized with *P. indica* exposed to abiotic stress conditions enhanced the peroxidase enzyme activity to mitigate the stress (Sun *et al*., 2010). Peroxidase activity was increased in *Pseudomonas aeruginosa* applied plants against *Soybean stunt virus* in soybean (Khalimi and Suprapta, 2011).

Leaf extracts of *B. spectabilis* treated tobacco plants produced more peroxidase against the tobamoviruses compared to untreated plants (Madhusudhan *et al.,* 2011). They also reported that neem oil and phyllanthus oil induced peroxidase activity and reduced the concentration of virus in treated plants. Hanaa (2017) studied the defense enzyme activities in plants infected with *Potato virus Y* using substances extracted from sea algae. The result revealed that there was increase in peroxidase, polyphenol oxidase, superoxide dismutase, catalayse and protein inhibitors in both pre and post treated plants than the infected plants.

Alex (2017) studied enzyme activity of *P. niruri* and *P. indica* colonized cowpea plants against BlCMV; and found that PO, PPO and PAL were increasing for particular period and subsequently declined in both pre-inoculated and post-inoculated treatments. Tomato plants treated with plant extracts against the TYLCV had shown a rapid increase in PO and PPO (El-Sawy *et al.*, 2018).

2.7.4. Analysis of PR proteins by SDS-PAGE

Prasad *et al.* (1995) analyzed the antiviral compounds of *C. inerme* and found two compounds of size 29 kDa and 34 kDa. *C. acuelateum* leaf extract was sprayed for the management of *Tobacco mosaic virus* in tobacco and *Sunhemp rosette virus* in *C. juncea* and its antiviral property was due to presence of 34 kDa proteins as deciphered in SDS-PAGE (Verma *et al.*, 1996). SDS-PAGE analysis of leaf extracts from *B. xbuttiana* yielded two proteins having molecular mass of 24 kDa (BBP24) and 28 kDa (BBP28) (Narwal *et al.*, 2001). Balasubrahmanyam *et al.* (2000) identified the antiviral proteins from *Celosia cristata* having molecular weight of 33 kDa and 37 kDa through SDS-PAGE but in electrophoresis and gel filtration, it represented 25 kDa and 27 kDa respectively.

Lohani *et al.* (2007) studied the viral suppression property of root extracts of *B. diffusa* against TMV in tobacco and related the protein of 30 kDa to the viral suppression. All the AVPs treated cowpea plants produced PR-proteins of molecular weight 24.5 kDa except *B. diffusa* treated plant and more PR-proteins were induced in all AVPs and *P. fluorescens* applied plants (Veena, 2007).

Najam *et al.* (2008) studied the viral resistance of *C. aculeatum* leaf extracts against the *Sunhemp rosette virus*. They identified a basic glycoprotein having molecular weight of 34 kDa which was responsible for inducing resistance against the virus. *P. indica* colonized plants produced additional protein bands compared to uninoculated plants which indicated the synthesis of PR proteins in treated *Helianthus annus* (Badge *et al.*, 2010). Electrolytic analysis of proteins in SDS-PAGE revealed that two proteins of 15 and 45 kDa were responsible for the control of BlCMV (Elsharkawy and El-sawy, 2015).

Hanaa (2017) found an increase in number of bands in algae extracted carrageenan treated plants infected with *Potato virus Y* in potato plants by SDS-PAGE analysis. They observed that the increase in number of proteins in treated plants was due to synthesis of defense related proteins. Electrophoretic analysis of AVP and *P. indica* treated plant produced new protein band indicating the synthesis of PR proteins in cowpea plants (Alex, 2017). *B. diffusa* treated plants induced PR-protein corresponding to molecular weight of 30 kDa whereas other treatments induced PR-protein of 36 kDa and 50 kDa (Sharma and Aswathi, 2017). Application of antiviral substances in bittergourd activated defense response in plants against begomoviruses due to the induction of novel proteins when examined in SDS-PAGE (Radhika, 2018).

Materials and Methods

3. MATERIALS AND METHODS

3.1 MAINTENANCE OF VIRUS AND *Piriformospora indica*

3.1.1. Maintenance of the virus

Blackeye cowpea mosaic virus was maintained in vegetable cowpea variety Sharika (*Vigna unguiculata var. sesquipedalis* (L.) Verdcourt and the local lesion host, *Chenopodium amaranticolor* by sap transmission. Young cowpea leaves showing typical symptoms like mosaic, leaf malformation and vein banding were collected from field and 1 g of leaf sample was homogenised in a pre-chilled mortar and pestle with 1.5 ml of 0.1 M Sodium phosphate buffer (pH 7.0) (Appendix I). The sap was filtered through muslin cloth and placed in ice box for immediate inoculation into selected cultivars. Primary two leaf stage of cowpea plants are susceptible to infection and 9 to 10 leaf stage of *C. amaranticolor* plants was selected for inoculation. *C. amaranticolor* was exposed to dark conditions prior to inoculation. Leaves were uniformly dusted with carborundum powder (600 mesh) and inoculation was done by dipping cotton in sap and gently rubbed on the dusted plant surface. After five minutes or prior to drying of sap, leaves were rinsed with distilled water using wash bottle.

3.1.2. Symptomatology

Young leaves showing mosaic and vein banding were collected and virus inoculum was transferred to cowpea and chenopodium plants by mechanical inoculation with 0.1 M Sodium phosphate buffer as described above and plants were maintained in insect proof glass house. Symptomatology was studied based on symptom developed in the mechanically inoculated cowpea and *C. amaranticolor* plants.

Based on the symptom expressed in the inoculated cowpea plants, Vulnerability index was calculated in accordance with the scale 0-5 developed by Bos (1982) as mentioned below,

0 = no symptom

1 = slight vein clearing, very little mottling of light and dark green colour in younger leaves – resistant (R)

2 = mottling of leaves with light and dark green colour – medium resistant (MR)

3 = blisters and raised surface on the leaves – medium susceptible (MS)

4 = distortion of leaves – susceptible (S)

5 = stunting of the plant with negligible or no flowering and fruiting – highly susceptible (HS)

Based on the above scale, rating was assessed and vulnerability index was calculated by using the equation,

$$VI = \frac{(0n1 + 1n2 + 2n3 + 3n4 + 4n5) \times 100}{nt\ (nc-1)}$$

VI - Vulnerability Index

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

nt - Total number of plants

nc - Total number of categories

3.1.3. 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 II). 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.

3.2. DETECTION OF BlCMV USING SEROLOGICAL METHOD

Serological based detection was done using polyclonal antibody developed specific to *Cowpea aphid-borne mosaic virus* (CABMV) and *Potato virus Y* (PV Y). Detection was done using Direct Antigen Coating - Enzyme linked immunosorbent assay (DAC-ELISA) and Dot immunobinding assay (DIBA).

3.2.1. Direct Antigen Coating-Enzyme linked immunosorbent Assay (DAC-ELISA)

ELISA procedure described by Huguenot *et al.* (1993) was followed for detecting the presence of BlCMV in infected cowpea plants.

One gram of young virus infected cowpea leaf was homogenized with 5ml of coating buffer with 2 per cent poly vinyl pyrolidone (PVP) in pre- chilled pestle and mortar. Similarly, healthy leaf collected from healthy plant was homogenized in similar conditions. Homogenate was centrifuged at 5000 rpm for 10 min at 4°C. From this, 200 µl of antigen was loaded in to immunological plates. The treatments included a negative sample, blank and positive sample. Each treatment was replicated thrice. It was incubated at 37°C for 2 h in an incubator. The wells were washed with Phosphate buffer saline-Tween (PBS-T) thrice at 3 min interval by ELISA plate washer. Blocking solution of 5 per cent spray dried milk (SDM) of 200 µl was dispensed and kept in incubation for 2 h at 37°C. The plate was washed thrice with PBS-T buffer as before. Subsequently, 200 µl of primary antibody (M/S. DSMZ, Germany) was added at 1:200 dilutions in PBS-T along with 2 per cent PVP and 0.2 per cent egg ovalbumin (PBS-TPO). It was incubated for 2 h at 37°C in an incubator. The plate was washed as before and 200 µl of secondary antibody conjugate with alkaline phosphate enzyme (M/s. DSMZ, Germany) at 1: 200 dilution in PBS-TPO was added. The plate was incubated at 37°C for 2 h. Wells were washed with PBS-T buffer as before in ELISA washer. Freshly prepared para-nitro phenyl phosphate in substrate buffer of 200 µl was loaded to each well and incubated it for 1 h at 37°C. The reaction was stopped by

adding 100 µl of 4 per cent sodium hydroxide. The colour development in the well was measured at 405 nm in an ELISA reader (Appendix III).

3.2.2. Dot-immunobinding Assay (DIBA)

The presence of BlCMV in infected cowpea sample was detected by Dot immunobinding assay by using polyclonal antibody of PVY and CABMV.

One gram of leaf sample was homogenized with 5 ml of antigen extraction buffer, filtered through muslin cheese cloth. Homogenate sample of 800 µl was transferred into eppendorf tube, and 400 µl of chloroform was added to it. The mixture was vortexed followed by centrifugation at 12000 rpm for 2 min. Supernatant of 200 µl was pipetted out, 800 µl of antigen extraction buffer was added and vortexed. Nitrocellulose membrane (NCM) in squares of 1x1 cm was taken, washed with tris buffer saline (TBS) and air dried. 10 µl of sample was spotted on to NCM and incubated for 30 min at room temperature. After incubation, it was washed and floated in blocking solution with gentle oscillation for 1 h at room temperature. NCM was rinsed with TBS buffer thrice at 10 min interval and air dried. NCM was floated in primary antibody of PVY and CABMV, which was diluted at 1:200 dilution in TBS-Spray dried milk (TBS-SDM) and incubated overnight at 4°C. NCM was rinsed with TBS buffer thrice as before and air dried. Secondary antibody (alkaline phosphatase conjugate) was dilute in 1:200 dilution in TBS-SDM and incubated for 1 h. It was rinsed with TBS buffer thrice followed by air drying. NCM was floated in freshly prepared solution of Nitro blue tetrazolium (NBT) and Bromo chloro-indolyl phosphate (BCIP) in substrate buffer and kept in dark for 30 min to develop colour. After colour development, NCM was rinsed in fixing solution for 10 min, air dried between Whatman filter paper sheets and the intensity of colour development observed in Gel Doc (Appendix IV).

3.3. STANDARDIZATION OF CO-CULTIVATION METHOD FOR FUNGAL ROOT ENDOPHYTE *P. indica*

In-vitro and *in-vivo* co-cultivation of *P. indica* with cowpea and *C. amaranticolor* were carried out as per the procedure described by Johnson *et al.* (2013).

3.3.1. *In vitro* co-cultivation of *P. indica* with cowpea and *C. amaranticolor* in Plant Nutrient Medium

Murashige and Skoog (MS) medium (Appendix V) was prepared, autoclaved and dispensed into sterilized jam bottles and 25 cm diameter petri plates. Cowpea and *C. amaranticolor* seeds were surface sterilized with 0.1 per cent mercuric chloride for 5 min, followed by three washing with sterile water and placed in jam bottles and petri plates respectively. This was placed at 24°C under controlled environment for germination of seeds. Plant nutrient medium (PNM) (Appendix VI) was slightly modified to facilitate the growth of both fungus and plants. *P. indica* from two weeks old culture was inoculated into solidified PNM media in jam bottles and petri plates and incubated in dark for growth of fungus. Twelve days old seedling of cowpea and *C. amaranticolor* was transferred into fungal lawn in jam bottles and petri plates respectively. This was placed in 24°C for colonization.

3.3.2. *In vitro* co-cultivation of *P. indica* with cowpea and *C. amaranticolor* in PNM

P. indica was inoculated in to jam bottles and petri plates contained PNM medium were incubated in dark for growth of fungus. Cowpea and *C. amaranticolor* seeds were surface sterilized with 0.1 per cent mercuric chloride followed by three washing in sterile water, air dried and placed in two weeks old culture of *P. indica* in PNM. It was maintained in controlled condition for colonization.

3.3.3. *In vivo* co-cultivation of *P. indica* with cowpea and *C. amaranticolor* in vermiculite-perlite medium

Vermiculite-perlite was used as planting medium for co-cultivation of cowpea and *C. amaranticolor* with *P. indica*. Potting medium was prepared by mixing 3 parts of vermiculite with one parts of perlite by volume. This medium was slightly moistened and sterilized for three consecutive days. Fungal disc of *P. indica* was inoculated into 100 ml potato dextrose broth in 250 ml conical flask. This was incubated for 18 days in room temperature in orbital shaker at 40 rpm. Mycelial mass was filtered through four layered muslin cloth and washed with sterile water. Mycelial mass of 1 per cent (w/v) was added to the sterilized vermiculite-perlite media, mixed uniformly and filled in portray of 5 cm width and 5 cm depth. Seeds of *C. amaranticolor* and cowpea were surface sterilized with 0.1 per cent mercuric chloride for 5 min followed by three washing in sterile water and placed in portrays. Portrays was maintained in growth chamber and sterile water sprinkled twice a day.

3.3.4. Root colonization by *P. indica*

Roots of cowpea and *C. amaranticolor* were collected at 7, 14 and 21 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 1cm length and transferred into test tube containing 5ml of 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. Again root bits were washed once with water and transferred into lactophenol tryphan blue for 2 min to stain the fungus in roots. The root bits were observed in microscope (Leica – ICC50 HD) for the presence of chlamydospores and colonization in each root bit. The percentage of root colonization was calculated as per Krishnaveni *et al.* (2015).

$$\text{Percentage of root colonization} = \frac{\text{No of root bits with colonization} \times 100}{\text{Total no. of roots bits observed}}$$

3.4. MANAGEMENT OF BLCMV USING PLANT EXTRACTS, *P. indica* AND ITS EXUDATES IN *C. amaranticolor*

3.4.1. Screening of AVPs

Plants having antiviral principles were examined against BlCMV in *C. amaranticolor*. Ten plants belonging to different families having potential antiviral principles were selected (Table 1). Experiments were laid out in CRD with three replications. In Pre-inoculation, above aqueous plant extracts were sprayed 24 h prior to inoculation of virus and aqueous plant extracts were sprayed 24 h after the inoculation of virus for post-inoculation.

Table 1: Botanicals having AVPs used for bioassay in local lesion host, *C. amaranticolor* against BlCMV

Sl. No.	Botanicals having antiviral principles
1	*Phyllanthus niruri*
2	*Boerhavia diffusa*
3	*Datura metel*
4	*Prosopis juliflora*
5	*Punica granatum*
6	*Eucalyptus globulus*
7	*Cinnamomum cassia*
8	*Phyllanthus emblica*
9	*Moringa oleifera*
10	*Vitex trifolia*

Table 2: Concentrations of AVP's used for bioassay in local lesion host, *C. amaranticolor* against BlCMV

AVPs	Treatments	Concentrations (%)		
Phyllanthus niruri	Pre-inoculation	1.00	0.50	0.25
	Post-inoculation	1.00	0.50	0.25

Similarly, treatments were applied to all the plant extracts on chenopodium against BlCMV. Days for symptom appearance, number of lesions and size of lesions were recorded for determining the efficiency of plant extracts. Virus inhibiting per cent was calculated by using the formula

$$\text{Per cent inhibition} = \frac{\text{C-T x 100}}{\text{C}}$$

C – Number of lesions on control leaves

T – Number of lesions on treated leaves

3.4.2. Evaluation of the *P. indica*-primed *C. amaranticolor* against BlCMV

Chenopodium plants were raised in double sterilized potting medium containing vermiculite and perlite in the ratio 3: 1. One gram of *P. indica* mycelial mass was added to the sterilized vermiculite-perlite mixture and germinated chenopodium seedling were transferred for colonization. A pot culture experiments was laid out in CRD with five replications. After 21 days, virus was inoculated to fully opened leaves by mechanical inoculation. Days for symptom appearance, size of lesions and number of lesions were recorded to determine virus inhibiting efficiency and the per cent inhibition was calculated by using the formula

$$\text{Per cent inhibition} = \frac{\text{C-T x 100}}{\text{C}}$$

C – Number of lesions on control leaves

T – Number of lesions on treated leaves

3.4.3. Evaluation of *P. indica*-water diffusible exudates (Pi-WDE) on *C. amaranticolor* against BlCMV

An experiment was laid out in CRD with pre- and post-inoculation, each treatment having three replications. 100 ml of potato dextrose broth was prepared

in 250 ml conical flask and sterilized at 121°C for 20 min. Fungal disc of 5 mm size *P. indica* was inoculated into the broth and incubated at room temperature for 21 days in 40 rpm in rotary shaker. Mycelial mass was strained through four layered muslin cloth and the mycelium was washed with sterile water. It was transferred into 250 ml conical flask containing 100 ml of sterile water and kept in rotary shaker at 40 rpm in room temperature. After 1, 3, 5, 7 and 10 days, the water was filtered through four layered muslin cloth, concentrated to 100 percentage (V/W of mycelium) at 40°C in rotary vacuum flash evaporator (Heidolph), filtered through syringe filter (0.22 µm) and sprayed to plants. Days for symptom appearance, number of lesions and size of lesions were observed. Above formula is used to calculate the per cent inhibition of virus.

3.5. EVALUATION OF THE AVP, *P. indica* AND Pi-WDE AGAINST BLCMV IN COWPEA

A pot culture experiment was conducted in CRD with three replications. AVPs and Pi-WDE having high efficiency of inhibiting BlCMV in local lesion host *C. amaranticolor* were selected for the bioassay in cowpea plants. Antiviral property of *P. niruri* (1 %) and *B. diffusa* (1 %); twelve days *P. indica*-primed cowpea seedlings and Pi-WDE of three days were used for the evaluation. Plant extracts and Pi-WDE was applied to cowpea plants at primary cotyledonary leaf stage in 24 h prior and after sap transmission of virus. Cowpea seeds were sown directly into potting mixture mixed with fungal mycelium and pre-inoculation was done on twelve days old seedlings. For post-inoculation, virus was inoculated at two leaf stage and was later transplanted to potting mixture with fungus. Control plant for each treatment was maintained separately. Days taken for symptom expression, vulnerability index, and roots and shoot biomass were accounted for observation.

3.6. PHYSICAL AND CHEMICAL PROPERTY OF AVP'S

Physical and chemical properties of the best two AVPs were analysed against BlCMV in local lesion host, *C. amaranticolor*. Days for symptom

appearance, number of lesions and size of lesions were recorded in all the experiments.

3.6.1. Thermostability of AVPs

Plant sample of one g was homogenised in pestle and mortar with distilled water and made up the volume into 100 ml. This was filtered with muslin cloth and the filtrate was sterilized at 121°C for 30 min and sprayed to *C. amaranticolor* with pre-inoculation to determine its heat stability.

3.6.2. Photolysis of AVPs

Leaf sample of five g was extracted with 500 ml of distilled water, filtered through muslin cloth and the filtrate was exposed to natural light from morning to evening. The cleared solution was sprayed onto *C. amaranticolor* with pre-inoculation to analyse its sensitiveness to light.

3.6.3. Chemical nature of AVPs

Plant extracts of protein and non-protein fraction was separated by methanol-chloroform method by Wessel and Flugge (1984). Plant sample was extracted by grinding 10 g of leaf sample in pestle and mortar with distilled water. The extract was filtered through cheese cloth and the final volume made into 10 ml. The extract was centrifuged at 6000 rpm for 15 min. 5 ml of supernatant was added with 15 ml of methanol and 5 ml chloroform; and vortexed. The appearance of extract changed into white cloudy, and spinned at 14000 rpm for 3 min. Protein fraction was present in between the upper aqueous methanol layers and lower chloroform layer. Upper aqueous layer was decanted into another vial constituting non-protein part. Fifteen 15 ml of methanol was added to the protein fraction, vortexed and again spinned at 14,000 rpm for 3 min to precipitate the protein. The supernatant was pipetted out and mixed with previous aqueous extracts and it constitutes the non-protein part of plant extracts. Protein pellet was dissolved in distilled water and diluted to 1 per cent concentration. The protein and non-protein fraction were sprayed separately into *C. amaranticolor* followed by BlCMV inoculation to find the active antiviral principles of the plant extract.

3.6.4. Molecular size

Ten gram of AVPs was homogenized with distilled water, filtered through muslin cloth and final volume was adjusted with 10 ml. It was centrifuged at 6000 rpm for 20 min; supernatant was filtered through Whatsmann filter paper and syringe filter. The final filtrate was passed through molecular sieve of 10 kDa and then through 1 kDa sieve. The fractions retained in 10 kDa sieve, fractions retained and passed through 1 kDa sieve were applied to plants to identify the size of active principle present in the plants.

3.7. ELUCIDATION OF BIOCHEMICAL MECHANISM INVOLVED IN THE RESISTANCE / TOLERANCE OF AVP- OR *P. indica*- OR Pi-WDE-TREATED COWPEA AGAINST BLCMV

Biochemical mechanism exhibited by healthy, infected and treatment applied plants were carried out in CRD with three replications. Cowpea variety Sharika was used for the study. Seeds were raised in pots and virus was mechanically transmitted at two leaf stage. Treatments were applied as pre- and post- applications. Biochemical changes were analysed by collecting samples at 5, 10, 15 and 30 days after virus inoculation. It included analysis of total soluble protein, total phenol, peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and pathogenesis related (PR)-protein by SDS-PAGE.

3.7.1. Total soluble protein

Total soluble protein was estimated by a method described by Bradford (1976). One-gram leaf sample was homogenised in 10 ml of 0.01 M sodium acetate buffer (pH-4.7) (Appendix VII), centrifuged at 5000 rpm for 15 minutes at 4°C. 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 VII). 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.

3.7.2. Total phenol

Total phenol was examined by a method described by Vidhyasekaran *et al.* (1992). Five hundred mg of leaf was boiled with 10 ml of 80 per cent ethanol for 10 min. The extract was filtered and re-extracted four times with 80 per cent ethanol. All the extracts were collected and air dried to evaporate the ethanol. Residues was dissolved in 2 ml of 80 per cent ethanol and used for phenol estimation. Reaction mixture contained 100 µl of sample, 500 µl of folin-ciocalteau reagent, 1 ml of saturated sodium carbonate and made up the volume to 3 ml with distilled water. It was kept in boiling water bath for 5 min and cooled in running tap water. The phenol content was recorded at 725 nm using spectrophotometer. Standard was prepared by dissolving 100 mg of catechol in 100 ml of distilled water.

3.7.3. 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 VIII) with a pinch of polyvinyl pyrrolidone in chilled pestle and mortar. It was filtered with cheese cloth and centrifuged at 6,000 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.

3.7.4. Polyphenol oxidase

Polyphenol oxidase (PPO) activity was assessed by following the procedure described by Mayer *et al.* (1965). One gram of leaf was grinded in 5 ml of sodium phosphate buffer (pH 6.5) (Appendix VIII) with Polyvinyl 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.

3.7.5. Phenylalanine ammonialyase

Phenylalanine ammonialyase (PAL) was analysed by using a procedure developed by Brueske (1980). Five hundred mg of leaf sample was homogenised in 15 ml of 0.01 M of sodium borate buffer (pH 8.8) (Appendix VIII) in chilled pestle and mortar. The extract was centrifuged at 4000 rpm for 10 min at 4°C and supernatant was saturated with 30 per cent ammonium sulphate. It was further centrifuged at 7200 rpm for 10 min and supernatant was again saturated with 70 per cent ammonium sulphate. It was centrifuged; pellet was resuspended in 9 ml of borate buffer and incubated overnight at 4°C. Reaction mixture consists of 1 ml of sodium borate buffer, 1 ml of enzyme extract and 1ml of phenylalanine. It was incubated at 32°C for 60 min. Reactions were stopped by adding 0.5 ml of trichloroacetic acid and incubate at 37°C for 5 min. The absorbance was read at 270 nm in spectrophotometer. PAL activity was expressed as micrograms of cinnamic acid produced per min per gram on fresh weight basis.

3.7.6. Analysis of pathogenesis related (PR) proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Induction of PR-protein in cowpea leaves were carried out in electrophoretic separation procedure as described by Laemelli (1970). Cowpea leaves from healthy, virus inoculated and treated plants were used for analysis.

Leaf sample of 500 mg from each were homogenised in 750 µl of cold denaturing solution in pre-chilled pestle and mortar. It was centrifuged at 5000 rpm for 15 min at 4°C. The supernatant was mixed with four times of cooled

acetone and the protein was allowed to precipitate by placing the mixture at 4°C for 30 min. It was subjected to centrifuge at 5000 rpm for 15 min at 4°C. The precipitate was resuspended in 100 µl of cold denaturating solution and 10 µl sample buffer; vortexed and centrifuged at 5000 rpm for 15min at 4°C. Supernatant was pipetted out and 10 µl of sample buffer and 10 µl denaturing solution were added and placed it in boiling water bath for 3 min and was used for SDS-PAGE. Molecular weight markers of 10 µl mixed with 10 µl of sample buffer was loaded. The protein concentration of each sample was adjusted to strength of 100 µg of protein following Bradford method.

Separating gel was prepared by mixing separating gel buffer stock of 5 ml with 8 ml of Acrylamide stock, 6.7 ml of double distilled water and 100 µl of sodium dodecyl sulphate (SDS) solution. The solution was mixed well and incubated for 3 min to liberate the dissolved gas in that mixture. It was followed by immediate adding of freshly prepared 10 per cent of ammonium per sulphate of 10 µl and 10 µl of tetra methyl ethylene diamine (TEMED). The solution was mixed uniformly and casted immediately between the glass plates. A layer of water was added above the polymerizing solution to quicken the polymerization process.

Stacking gel was prepared by adding stacking gel buffer stock of 2.5 ml with SDS 100 µl, acrylamide stock of 1.3 ml and double distilled water of 6.1 ml. This solution was mixed uniformly and incubated for a minute to remove the gas. The water layer above the separating gel was removed and washed with electrode buffer. The comb was placed in position and then prepared stacking gel was poured over the polymerized separating gel.

After polymerisation, the samples were loaded into wells. Initially electrophoresis was performed at 65 V till the tracking dye reached the separating gel. The voltage was increased to 100 V and maintained till the dye reached the bottom of separating gel. The gel was removed and incubated in staining solution for overnight with uniform oscillation. Later the gel was transferred into destaining solution. The proteins present in the sample were appeared as bands in

gel according to the corresponding molecular weight. The gel was observed in BIORAD Molecular Imager (Gel Doc). The molecular weight of new and induced polypetides was calculated from the standard graph prepared for a 12 per cent gel using standard markers (Appendix IX).

Results

4. RESULTS

The present study on "Management of *Blackeye cowpea mosaic virus* using natural products from botanicals and the fungal root endophyte *Piriformospora indica*" was conducted during the period 2017-2019 at the Department of Plant Pathology, College of Agriculture, Vellayani to utilize natural products from potential antiviral plant extracts and the root endophyte *Piriformospora indica* for the management of the virus. The results of the study are detailed here.

4.1 MAINTENANCE OF THE VIRUS AND *Piriformospora indica*

4.1.1 Maintenance of the virus

Blackeye cowpea mosaic virus (BlCMV) was maintained in systemic host, vegetable cowpea variety Sharika and the local lesion host, *Chenopodium amaranticolor* by mechanical transmission using 0.1 M Sodium phosphate buffer (pH 7.0). All the inoculated cowpea seedlings expressed symptom on trifoliate leaves at 7th day after inoculation (DAI) and on 5th day for *C. amaranticolor*.

4.1.2 Symptomatology

Inoculated cowpea plants initially produced vein clearing or vein banding symptoms on the emerged first trifoliate leaves. Vein clearing symptoms were also observed in seedling raised from seeds of virus infected plants. The typical symptoms included mosaic, leaf blistering, vein netting, reduction in leaf size and malformation. Severely infected plant was stunted with floral malformation and reduced pod length with few seeds (Plate 1). Symptoms produced on mechanically inoculated plants were similar to symptoms under natural field conditions. In *C. amaranticolor*, local lesion appeared as minute spots on fourth day and yellow chlorotic localized lesions of 2 to 3 mm in size was observed on 5th DAI (Plate 2)

4.1.3 Maintenance of fungal root endophyte *P. indica*

The beneficial fungal root endophyte *P. indica* was maintained in potato dextrose agar (PDA) and potato dextrose broth (PDB) by continuous sub-culturing at every fifteen days interval. The fungus attained 9 cm radial growth after nine

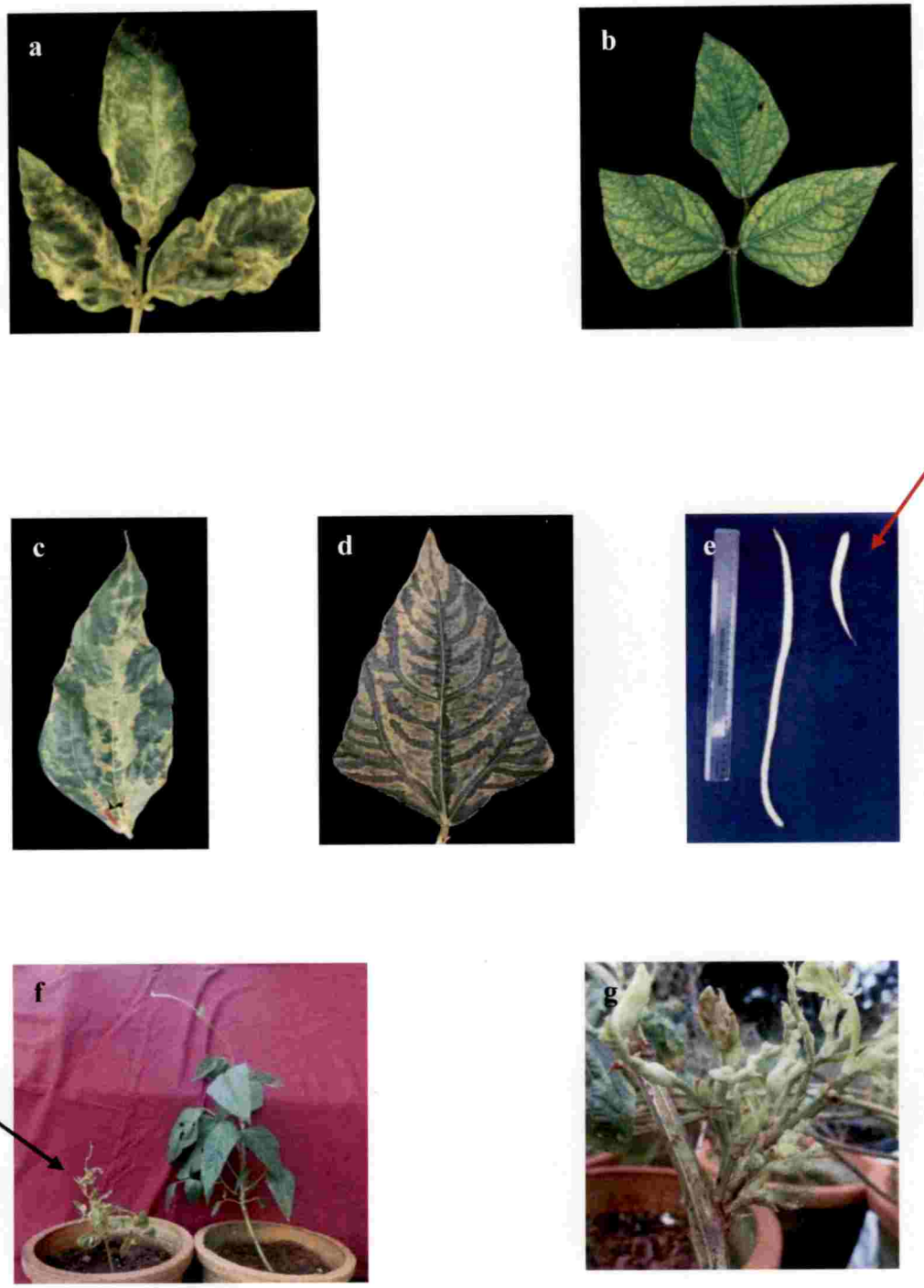


Plate 1. Common symptoms of BlCMV in vegetable cowpea. (a) Leaf blistering; (b) vein netting; (c) mosaic; (d) Vein banding; (e) pod length reduction; (f) stunting; (g) flower malformation.

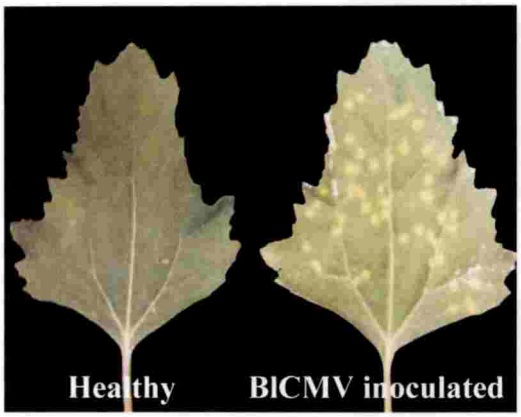


Plate 2. Local lesions symptom of BlCMV in *Chenopodium amaranticolor.*

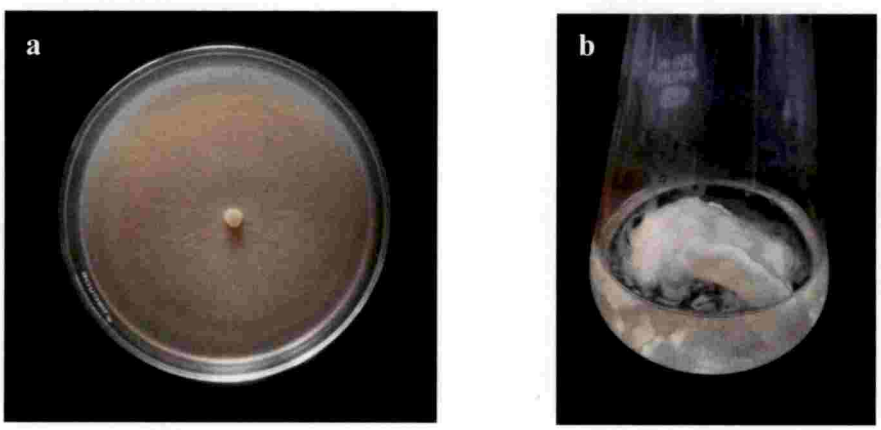


Plate 3. Maintenance of *Piriformospora indica* in potato dextrose medium
(a) Radial growth of *P. indica* in potato dextrose agar on 10th day;
(b) Growth of *P. indica* in PD broth on 15th day.

days of inoculation in petri plate containing PDA (Plate 3a) and it took twenty one days to form mycelial mat on 100 ml PDB in 250 ml conical flask (Plate3b).

4.2 SEROLOGICAL DETECTION OF *Blackeye cowpea mosaic virus*

Protein based techniques like ELISA and DIBA were employed for the serological detection of the virus.

4.2.1 Enzyme linked immunosorbent assay (ELISA)

Direct antigen coating Enzyme linked immunosorbent assay (DAC-ELISA) was used to detect the virus from the virus infected samples. Sap extracted from the cowpea leaves was used as antigen source. Polyclonal antibodies of *Cowpea aphid-borne mosaic virus* (CABMV) and *Potato virus Y* (PVY) were used for the study since BlCMV belongs to potyvirus and the polyclonal antibody of BlCMV was not commercially available. Absorbance was measured at 415 nm in an ELISA reader. Results are summarised in the Table 3 and Table 4.

BlCMV infected leaf sample were found positive to polyclonal antibodies of CABMV and PVY by colour development (Plate 4). The absorbance values for infected samples showing varying symptoms of mosaic, vein banding and vein clearing were 1.415, 1.434 and 1.435 respectively with CABMV antiserum. The OD values of the infected samples were more than 3 times compared to healthy samples. OD values of 1.457, 1.431 and 1.458 respectively were obtained for the infected samples with symptoms of mosaic, vein banding and vein clearing when treated with polyclonal antibody for PVY and the absorbance values were more than 3.5 times over healthy control.

4.2.2 Dot immunobinding assay (DIBA)

Cowpea leaves with typical viral symptoms were used to detect the presence of BlCMV by using DIBA. It was observed that, the sample reacted positively to polyclonal antibodies of CABMV and PVY by the development of definite purple colour spots on nitrocellulose membrane for the infected samples (Plate.5) whereas no colour development was observed in healthy and buffer control. Intensity of colour development was calculated using GelDoc and the

Table 3. Reaction of BlCMV to polyclonal antibody of CABMV by DAC-ELISA

Sl. No	Samples	Mean OD values	Fold increase in OD values over healthy	Reaction
1	Healthy	0.466	-	-
2	Mosaic	1.415	3.03	+
3	Vein banding	1.434	3.07	+
4	Vein clearing	1.435	3.07	+

Table 4. Reaction of BlCMV to polyclonal antibody of PVY by DAC-ELISA

Sl. No	Samples	Mean OD values	Fold increase in OD values over healthy	Reaction
1	Healthy	0.382	-	-
2	Mosaic	1.457	3.81	+
3	Vein banding	1.431	3.74	+
4	Vein clearing	1.458	3.81	+

+ Presence of virus

- Absence of virus

Table 5. Reaction of BlCMV to polyclonal antibody CABMV in infected cowpea plants

Sl. No.	Label	Type	Volume (Int)	Adj. Vol. (Int)	Mean Bkgd. (Int)	Min. Value (Int)	Max. Value (Int)	Mean value	Std. Dev.	Area (mm^2)
1	U1	Diseased	11,366,171	2,901,465	915.2	671	2,554	1,228.9	418.9	43.1
2	U2	Healthy	9,079,183	1,535,965	878.7	623	2,080	1,057.6	224.4	40.0
3	B1	Background	9,458,327	29,677	827.0	638	1,445	829.6	62.6	44.1

Table 6. Reaction of BlCMV to polyclonal antibody PVY in infected cowpea plants

Sl. No.	Label	Type	Volume (Int)	Adj. Vol. (Int)	Mean Bkgd. (Int)	Min. Value (Int)	Max. Value (Int)	Mean value	Std. Dev.	Area (mm^2)
1	U1	Diseased	19,952,473	6,641,211	1,980.5	1,120	3,707	2,968.7	394.3	28.6
2	U2	Healthy	8,930,304	1,098,520	1,276.2	1,018	3,070	1,455.2	272.0	28.6
3	B1	Background	7,938,770	11,846	1,291.7	1,085	2,857	1,293.6	109.6	28.6

Plate 4. Detection of BlCMV using polyclonal antibodies of CABMV and PVY in DAC-ELISA

(C3, C4, C5, E3, E4, E5 (infected sample) – CABMV; C9, E9 (Healthy) – CABMV; C6, C7, C8, E6, E7, E8 (infected sample) – PVY; C10, E10 (Healthy) – PVY)

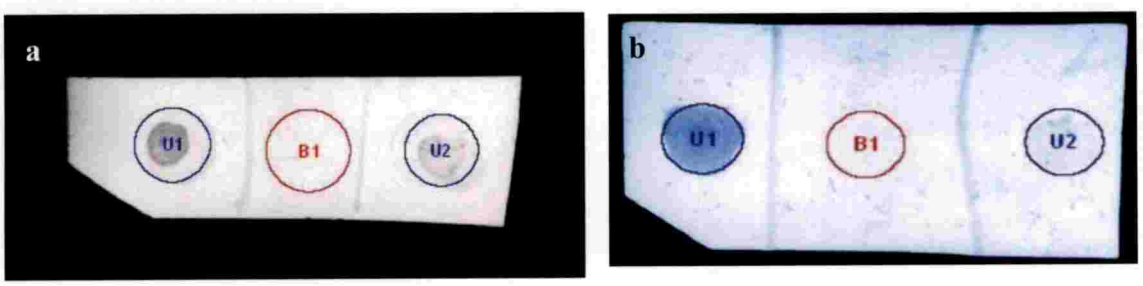


Plate 5. Postitive reaction of BlCMV to polyclonal antibodies (a) CABMV and (b) PVY in DIBA.

mean value of the colour intensity was found to be higher in infected samples over healthy for both CABMV and PVY polyclonal antibodies (Table 5 and 6).

4.3 STANDARDIZATION OF CO-CULTIVATION METHOD FOR *P. indica* WITH COWPEA AND *C. amaranticolor*

4.3.1. *In vitro* co-cultivation of *P. indica* with cowpea and *C. amaranticolor*

Cowpea seedlings in cotyledonary leaf stage from MS medium were transferred into jam bottles containing PNM media with two weeks old fungal lawn of *P. indica*. Plants could not be survived in *in vitro* co-cultivation. Hence cowpea seeds were directly raised on two weeks old mycelium net of *P. indica* in jam bottles containing PNM medium. Mycelium was observed in roots after five days of co-cultivation and chlamydospores were produced on root surface on seventh day after co-cultivation (Plate 6). On 14th day, chlamydospore clumped together on the root surface and form colonization within the roots.

In *C. amaranticolor*, germinated seedlings from MS media were planted on mycelial lawn of *P. indica* in petriplate. Plants were unable to regenerate. *C. amaranticolor* seeds were directly placed on fully grown *P. indica* mycelium of two weeks old culture in PNM medium. Chenopodium germinated in two weeks and colonization of the fungus was observed in roots at three weeks after co-cultivation (Plate 7). *In vitro* co-cultivation of *P. indica* with Chenopodium was difficult due to poor and non-uniform germination of *C. amaranticolor* seeds.

4.3.3 *In vivo* co-cultivation of plants and fungal endophytes in vermiculite and perlite media

Co-cultivation of cowpea and chenopodium with *P. indica* were conducted in portrays containing vermiculite-perlite (3:1) medium mixed with 1 per cent mycelium of *P. indica*. Roots were observed for colonization in intervals. In cowpea, mycelium was seen within roots after five days and chlamydospores on the root surface after seven days of co-cultivation (Plate 8). Chlamydospores were seen inside the colonized roots on 10th day after co-cultivation. The colonization

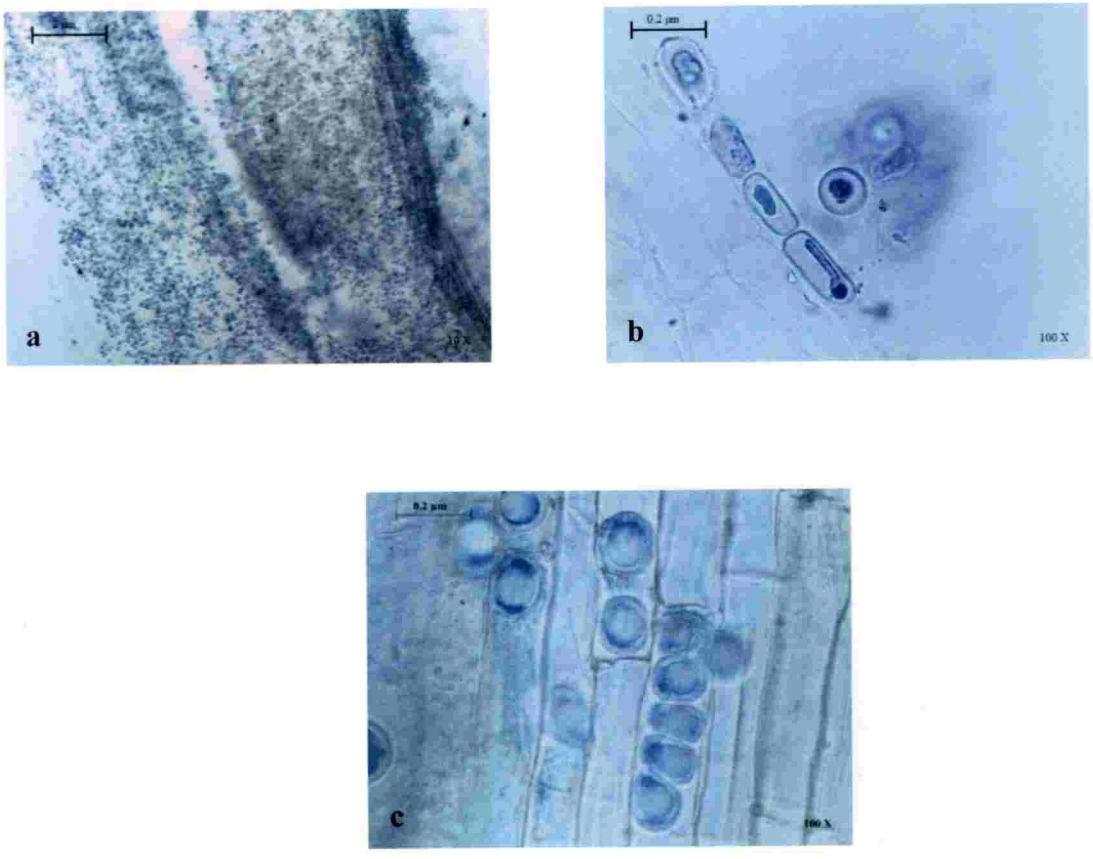


Plate 6. *In vitro* root colonization of *P. indica* in cowpea. (a) Chlamydospores in roots seven DAC; (b) Colonization in roots 14 DAC; (c) Colonization in roots 21 DAC.

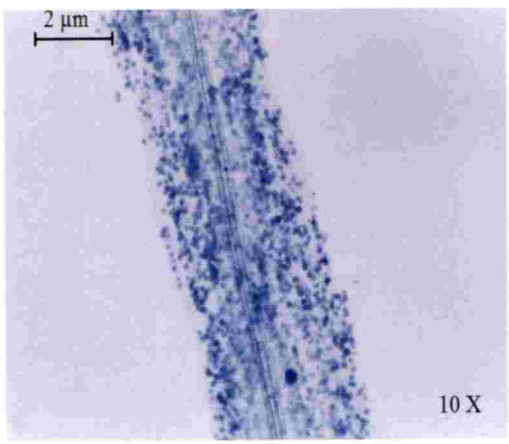


Plate 7. *In vitro* root colonization of *P. indica* in *C. amaranticolor*, three weeks after co-cultivation.

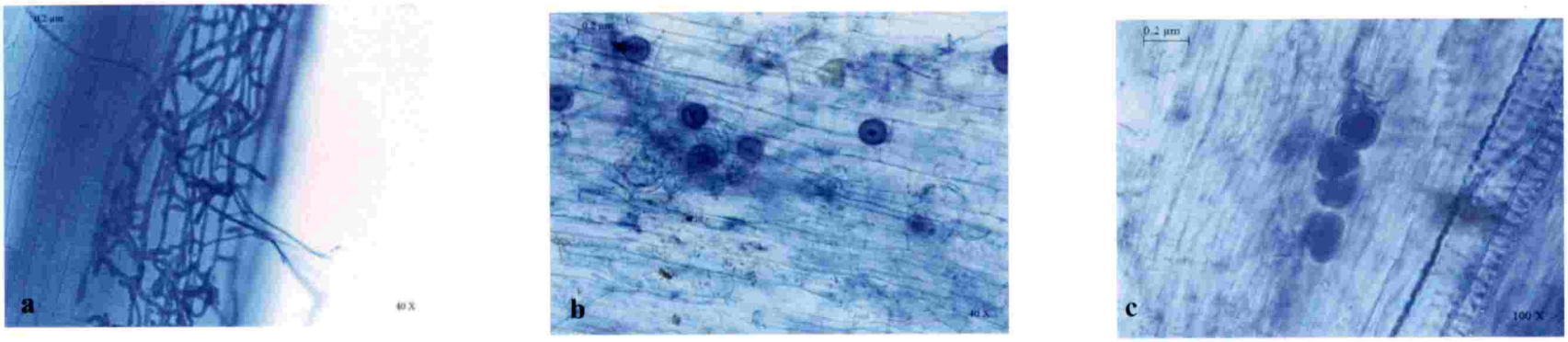


Plate 8. ***In vivo*** **root colonization of** ***P. indica*** **in cowpea. (a) Mycelium in roots five DAC; (b) Chlamydospores in roots seven DAC; (c) Colonization in roots 14 DAC.**

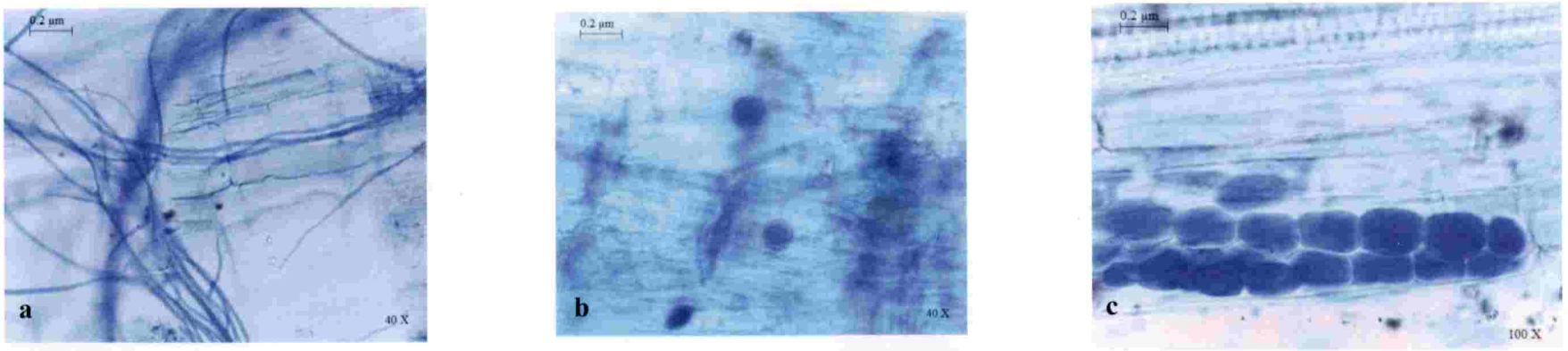


Plate 9. ***In vivo*** **root colonization of** ***P. indica*** **in** ***C. amaranticolor*****. (a) Mycelium in roots one WAC; (b) Chlamydospores in roots two WAC; (c) Colonization in roots three WAC.**

pattern was similar to previous method of colonization of cowpea with *P. indica* under *in vitro* condition.

In chenopodium, mycelium was observed in roots within a week of co-cultivation. Chlamydospores were produced on root surface within two weeks (Plate 9) and in three weeks inside the roots. Thus, *P. indica* takes more than 21 days for chlamydospore production in *C. amaranticolor* and 14 days in cowpea roots under *in vitro* and *in vivo.*

4.4 MANAGEMENT OF BLCMV USING BOTANICALS, FUNGAL ROOT ENDOPHYTE AND ITS PRODUCT ON *Chenopodium amaranticolor*

4.4.1. Screening of aqueous plant extracts having potential antiviral principles on BlCMV in *C. amaranticlor*

Plants having antiviral principles were screened for its antiviral activity against BlCMV in local lesion host, *C. amaranticolor* with three different concentrations for both pre- and post- inoculation. The results revealed that all the selected AVPs were effective in inhibiting / reducing the local lesions formed by BlCMV in *C. amaranticolor* at different concentrations tested. The per cent inhibition of virus was positively correlated with increase in concentration of leaf extracts. The per cent of inhibition of local lesions developed by BlCMV ranged from 23 to 86 in pre-inoculated treatments and 17 to 86 for post-inoculated treatments (Table 7).

P. niruri treated plants significantly inhibited the local lesions produced by BlCMV over control with the per cent of inhibition ranged from 44 to 86 for pre-inoculated plants and 39 to 86 for post-inoculated plants. Least number of local lesions were observed at one per cent concentrations in both the method of inoculation and the per cent of inhibition increased with increase in concentration of plant extracts with the average inhibition of more than 60 at 0.5 per cent and more than 40 at 0.25 per cent over the un-inoculated control (Table 7; Plate 10).

The effective plant extract next to *P. niruri* was *B. diffusa*. The per cent inhibition of local lesions of BlCMV over control by *B. diffusa* ranged from 54 to

Table 7. Screening of aqueous extracts of botanicals having potential antiviral principles against BlCMV in local lesion host, *C. amaranticolor*

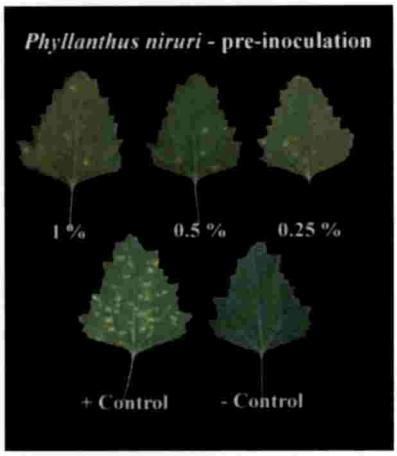
Botanicals with potential AVPs	Concentration (W/V)	Days for symptom appearance		Size of lesions (mm)		Percent inhibition over control		Number of lesion	
		Pre-I	Post-I	Pre-I	Post-I	Pre-I	Post-I	Pre-I	Post-I
Phyllanthus niruri	1.0 %	5	5	1.5	2.0	84.16	81.56	4.33±0.57	5.33±1.52
	0.5 %	5	5	2.0	1.5	64.05	61.60	9.33±1.52	10.33±1.54
	0.25 %	5	5	2.0	2.0	46.86	41.62	15.93±1.15	11.33±1.52
Boerhavia diffusa	1.0 %	6	6	1.5	1.5	79.13	75.16	5.66±0.57	6.33±1.154
	0.5 %	6	6	2.0	1.5	67.29	63.60	7.67±11.52	9±2
	0.25 %	6	5	1.5	1.5	61.08	59.41	12.67±2.51	13.67±1.15
Datura metel	1.0 %	5	5	1.5	1.5	63.28	58.49	12.67±2.51	13.67±1.15
	0.5 %	5	5	1.5	1.5	47.88	41.79	18±1	20±1
	0.25 %	5	5	2.0	1.5	33.42	32.46	22.67±1.52	23±1.73
Prosopis julifera	1.0 %	5	5	1.5	1.5	64.66	62.03	9.67±1.52	10±1.73
	0.5 %	5	5	1.5	2.0	49.93	48.63	13.67±1.15	13±0.57
	0.25 %	5	5	2.0	2.0	40.43	36.56	16±1	17±1
Punica granantum	1.0 %	5	5	2.0	2.0	67.52	61.98	11±1	12.67±1.52
	0.5 %	5	5	2.0	2.0	53.43	48.37	15.67±1.52	17.33±2.08
	0.25 %	5	5	1.5	2.0	31.35	28.86	23.33±1.52	24±2
Eucalyptus globulus	1.0 %	5	5	1.5	1.5	62.56	58.8	10.67±1.52	12±2
	0.5 %	5	5	1.5	2.0	50.08	46.18	14±1	15.33±0.57
	0.25 %	5	5	1.5	2.0	38.03	34.21	17.67±0.57	18.33±0.57
Cinnamomum cassia	1.0 %	5	5	1.0	1.5	68.79	63.63	8.33±1.52	10±1

	0.5 %	5	5	1.0	1.5	56.37	51.88	12±1	13.66±1
	0.25 %	5	5	1.0	1.5	48.91	44.38	14±1	15±1
Phyllanthus emblica	1.0 %	5	5	1.0	1.5	78.12	73.71	6.33±0.57	7±1
	0.5 %	5	5	1.0	2.0	55.11	53.32	12±1	12.33±1.52
	0.25 %	5	5	1.5	2.0	41.31	42.14	15.67±1.52	16±2
Moringa oleifera	1.0 %	5	5	1.5	1.5	73.40	68.56	7.33±1.15	8.67±1.15
	0.5 %	5	5	1.5	1.5	58.8	54.65	12±2	13±1
	0.25 %	5	5	2.0	2.0	36.33	32.74	17.67±1.52	19±1
Vitex trifolia	1.0 %	5	5	1.5	1.5	62.76	60.23	10.33±1.52	11±1
	0.5 %	5	5	1.5	1.5	42.83	40.14	16.33±1.52	17±1
	0.25 %	5	5	2.0	1.5	27.50	20.68	20.66±1.15	23.33±1.15
Control		5		2.0		100		29	
SE(m) ±						2.4		0.07	
CD (0.05)						6.62		2.18	

- Values are the mean of three replications ± standard deviation

Pre-I – Pre-inoculation

Post-I – Post-inoculation



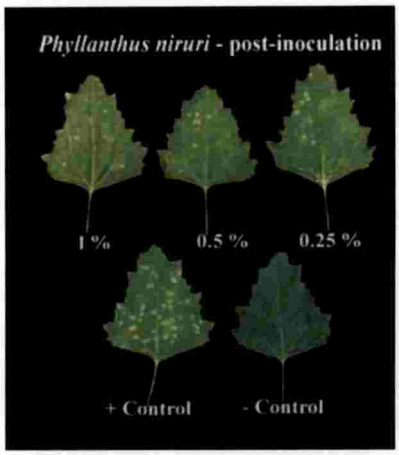
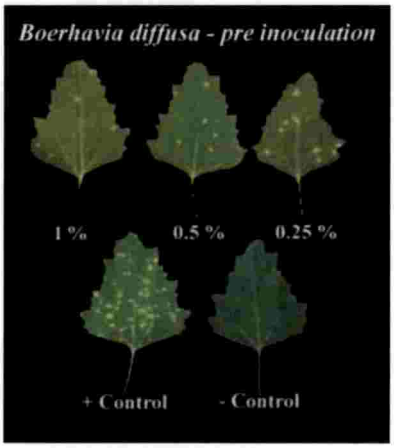


Plate 10. Effect of *Phyllanthus niruri* aqueous extracts on BlCMV in local lesion host, *C. amaranticolor.*



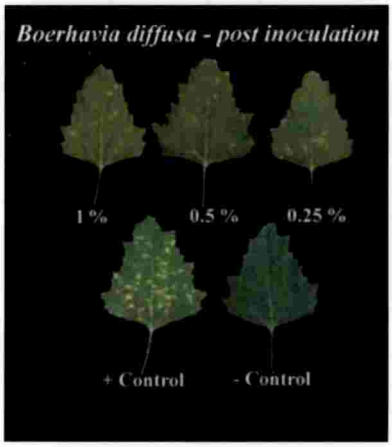
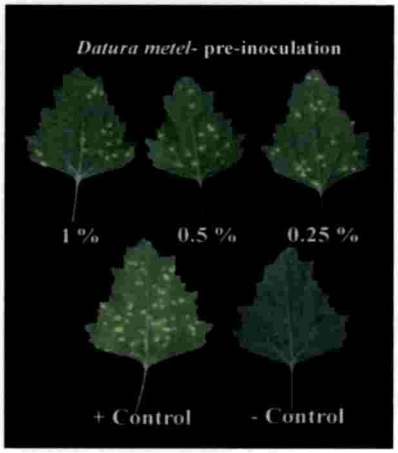


Plate 11. Effect of *Boerhavia diffusa* aqueous extracts on BlCMV in local lesion host, *C. amaranticolor.*



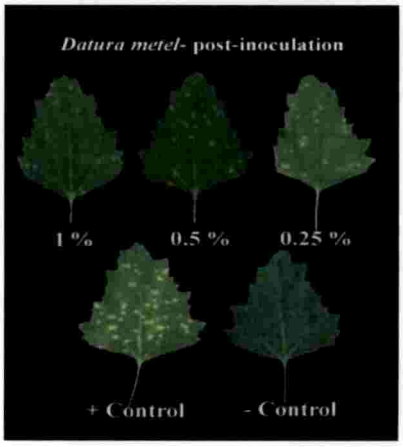
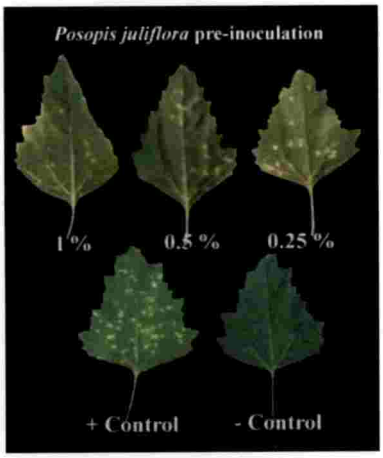


Plate 12. Effect of *Datura metel* aqueous extracts on BlCMV in local lesion host, *C. amaranticolor.*



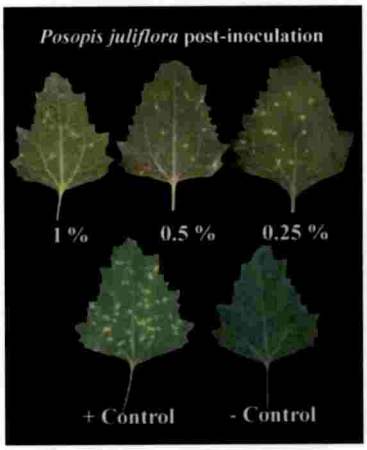
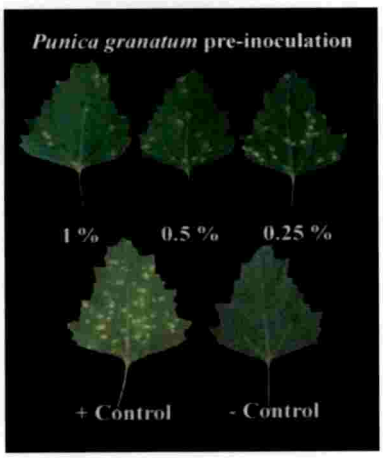


Plate 13. Effect of *Prosopis juliflora* aqueous extracts on BlCMV in local lesion host, *C. amaranticolor.*



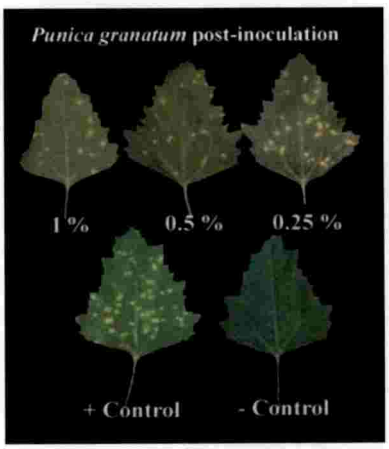
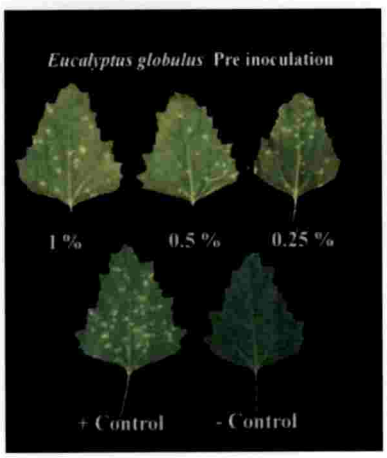


Plate 14. Effect of *Punica granatum* aqueous extracts on BlCMV in local lesion host, *C. amaranticolor.*



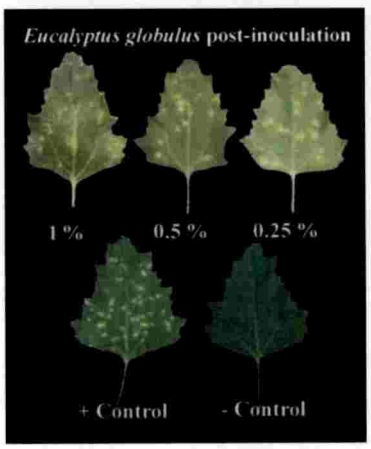
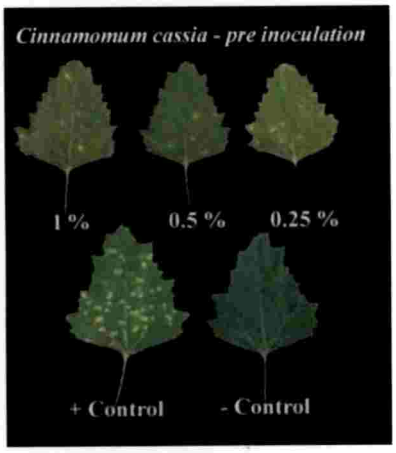


Plate 15. Effect of *Eucalyptus globulus* aqueous extracts on BlCMV in local lesion host, *C. amaranticolor.*



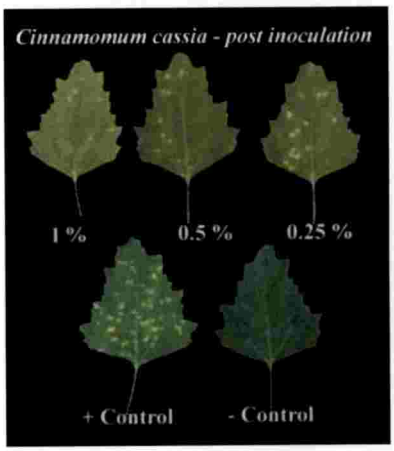
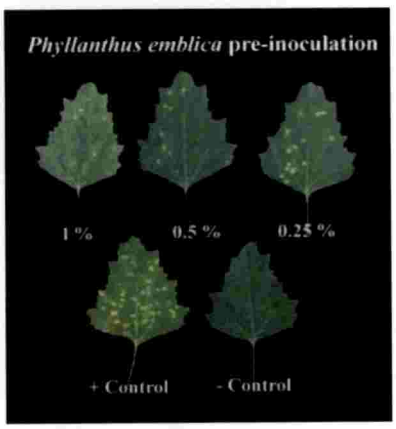


Plate 16. Effect of *Cinnamomum cassia* aqueous extracts on BlCMV in local lesion host, *C. amaranticolor.*



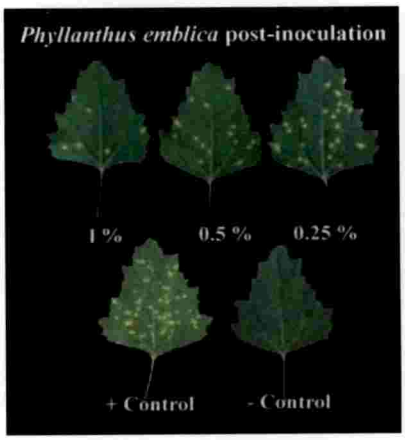
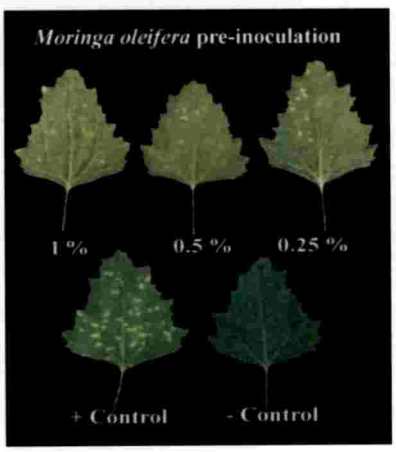


Plate 17. Effect of *Phyllanthus emblica* aqueous extracts on BlCMV in local lesion host, *C. amaranticolor.*



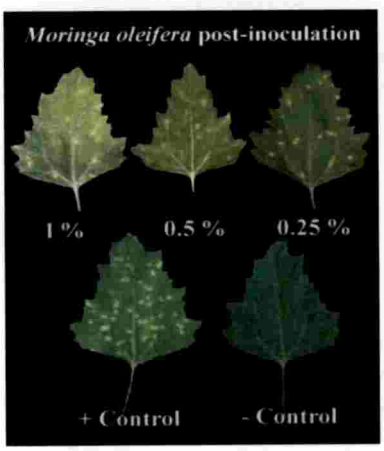
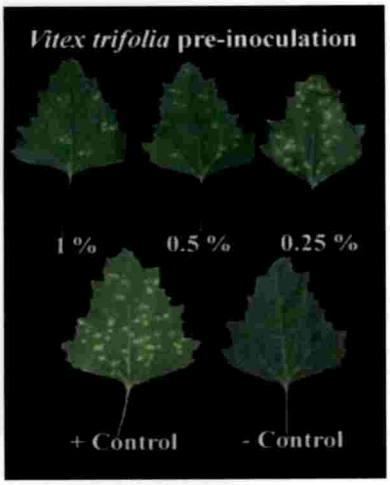


Plate 18. Effect of *Moringa oleifera* aqueous extracts on BlCMV in local lesion host, *C. amaranticolor.*



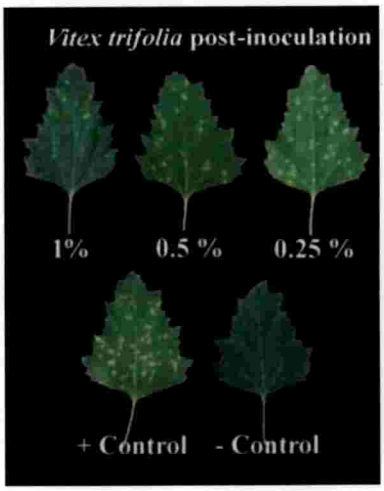


Plate 19. Effect of *Vitex trifolia* aqueous extracts on BlCMV in local lesion host, *C. amaranticolor.*

83 for pre-inoculated plants and 56 to 78 for post-inoculated plants. The per cent of inhibition was more than 60 over control even in lower concentration of 0.5 and 0.25 in both pre- and post- inoculated plants (Table 7; Plate 11). The results revealed that virus inhibition property was very significant with *B. diffusa* even at very low concentration.

The plants treated with AVPs from *D. metel* inhibited or reduced the local lesion developed by BlCMV with a per cent of inhibition over control ranged from 28 to 66 for pre-inoculation and 26 to 61 for post-inoculation (Table 7; Plate 12). The per cent inhibition of local lesions developed by BlCMV in *C. amaranticolor* treated with *Prosopis juliflora* over control ranged from 38 to 70 for pre- inoculated plants and 33 to 66 for post inoculated plants (Table 7; Plate 13).

Punica granatum was one of the effective botanicals having potential AVPs with the per cent inhibition over control ranged from 27 to 70 for pre-inoculated plants and 22 to 66 for post-inoculated plants (Table 7; Plate 14). The per cent inhibition of local lesions produced by the virus in chenopodium pre-inoculated with *Eucalyptus globulus* over control ranged from 35 to 68 and 30 to 52 for post-inoculated plants (Table 7; Plate 15). *Cinnamomum cassia* treated plants inhibited the local lesion developed by BlCMV with per cent inhibition over control ranged from 46 to 73 for pre-inoculation and 41 to 67 for post-inoculated plants (Table 7; Plate 16).

Phyllanthus emblica was the third most effective AVPs found among the selected plant extracts against BlCMV with the per cent inhibition over control ranged from 35 to 79 for pre-inoculated plants and 35 to 76 for post inoculated plants (Table 7; Plate 17). The next effective AVPs followed by *P. emblica* against BlCMV was *Moringa oleifera* with the per cent of inhibition over control ranged from 32 to 76 for pre-inoculated plants and 29 to 70 for post-inoculated plants (Table 7; Plate 18).

Vitex trifolia was one of the potential AVPs, the per cent of inhibition against BlCMV in *C. amaranticolor* was least when compared to other selected

AVPs. The per cent inhibition of local lesions over control ranged from 23 to 66 for pre inoculated and 17 to 62 for post-inoculated plants (Table 7; Plate 19).

Among the ten plant extracts screened against BlCMV *in C. amaranticolor,* the highest inhibition of the virus was recorded with *P. niruri* treated plants followed by *B. diffusa*, *P. emblica* and *M. oleifera* which inhibited the virus more than 70 per cent over control even at one per cent concentration (Table 7). Pre-treatment of the plants with AVPs was more effective than post-treatment application.

4.4.2. Evaluation of *P. indica*-primed *C.amaranticolor* against BlCMV

P. indica is a novel fungal root endophyte that enhances defense mechanism of plants against various biotic and abiotic stress. Its effectiveness against BlCMV in *C. amaranticolor* was evaluated. *P. indica* root colonization in *C. amaranticolor* plants significantly reduced the local lesions developed by BlCMV with the per cent inhibition ranging from 60 to 73 over control (Table 8; Plate 20).

4.4.3. Evaluation of *P. indica*-water diffusible exudates on BlCMV in *C. amaranticolor*

P. indica-water diffusible exudates (Pi-WDE) collected from different day-old exudates were tested against BlCMV in *C. amaranticolor* by pre- and post- inoculation methods. All the exudates significantly reduced the local lesions developed by BlCMV with the per cent inhibition over control ranged from 38 to 77 for pre-inoculation and 33 to 72 for post inoculation (Table 9, Plate 23). The highest inhibition of more than 70 per cent over control was observed on exudate from three day-exudates in sterile water compared to 1, 5, 7 and 10 day-old culture exudates (Table 9, Plate 23). Irrespective of the method of application, per cent inhibition of virus depended on the different day of culture in sterile water.

Table 8. Effect of *P. indica*-priming in local lesion host, *C. amaranticolor* against BlCMV

Treatment	Days for symptom appearance	Size of lesions (mm)	Number of lesions	Percent inhibition over control
P. indica alone	0	0	0	100.00
BlCMV alone	5	0.5	15.8±0.86	0.00
P. indica-primed seedlings + BlCMV	5	0.5	4.8±0.37	67.99±2.49
Control	0	0	0	100.00
SE (m) ±			1.4	3.73
CD (0.05)			0.46	1.24

- Values are the mean of five replications ± standard deviation

Table 9. Effect of *Pi*-WDE on BlCMV in local lesion host, *C. amaranticolor*

Exudates	Method of application	Days for symptom appearance	Size of lesions (mm)	Number of lesions	Per cent inhibition over control
1 days	Pre-inoculation	6	2	9.33	48.14
	Post-inoculation	6	2	8.66	51.85
3 days	Pre-inoculation	6	2	4.33	75.92
	Post-inoculation	6	2	5.33	70.36
5 days	Pre-inoculation	6	2	6.33	64.81
	Post-inoculation	6	2	6.67	66.66
7 days	Pre-inoculation	6	2	9.0	49.99
	Post-inoculation	6	2	8.33	53.7
10 days	Pre-inoculation	6	2	9.0	49.99
	Post-inoculation	6	2	11.33	38.88
Control		6	2	18.0	00.00
SE (m) ±				0.56	4.404
CD (0.05)				1.91	11.21

- Values are the mean of three replications

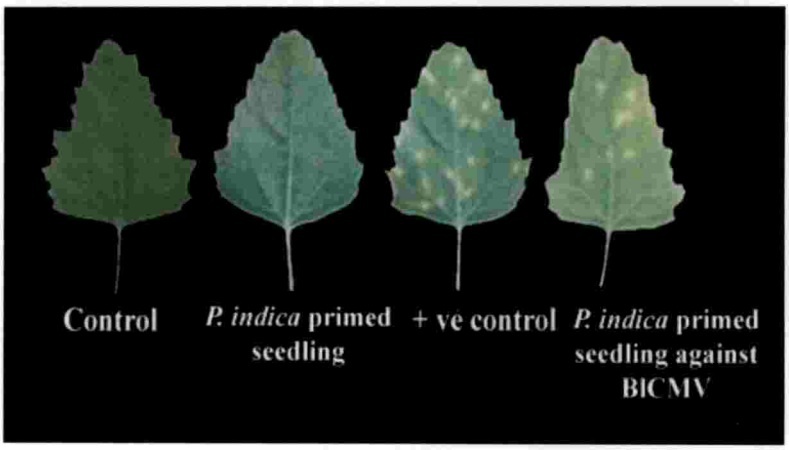


Plate 20. Effect of *P. indica* priming in *C. amaranticolor* leaves against BlCMV.

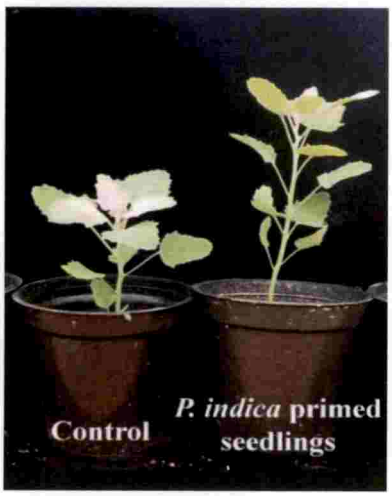


Plate 21. *P. indica*-primed *C. amaranticolor* seedlings.

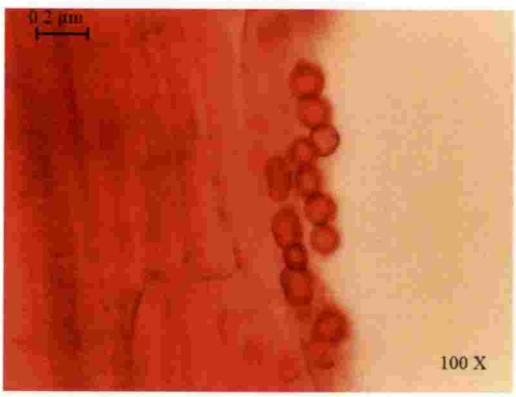


Plate 22. *P. indica* colonization in chenopodium roots.

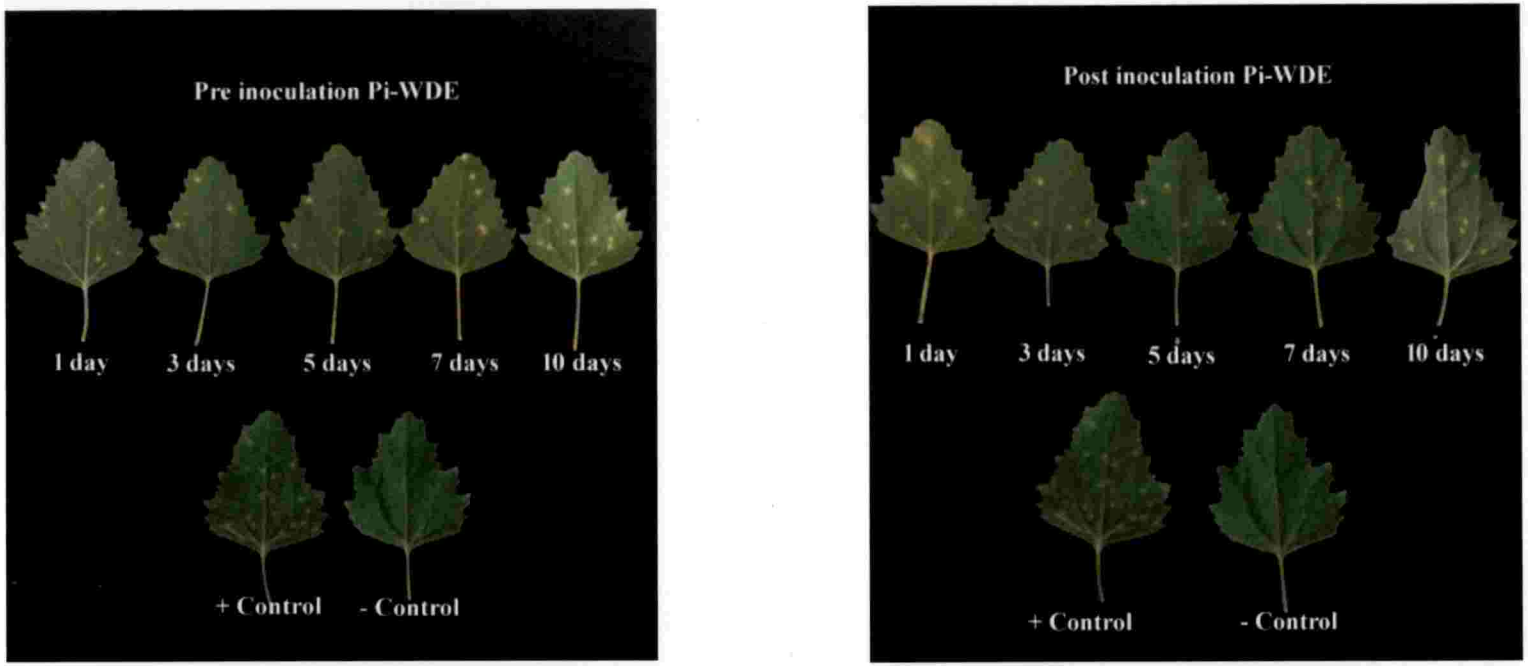


Plate 23. Effect of *P. indica*-water diffusible exudates on BlCMV in local lesion host, *C. amaranticolor*.

4.5. MANAGEMENT OF BlCMV USING PLANT EXTRACTS, *P. indica* AND ITS EXUDATES IN COWPEA

The plant extracts, Pi-WDE and *P. indica*-priming showed the highest inhibition of local lesion in *C. amaranticolor* were selected to find out its efficiency in cowpea against BlCMV. Vulnerability index was assessed and the highest reduction of the disease was recorded in *P. indica* colonized pants prior to virus inoculation (71 % over control) followed by *P. niruri* pre-treated plants (Plate 24; Plate 25). Treatments that were applied prior to virus inoculation showed less vulnerable index to the viral infection than the post-inoculation (Table 10; Plate 26; Plate 27).

Root and shoot biomass of treated plants were assessed and was found that the *P. indica*-primed plants inoculated with the virus yielded high root and shoot biomass than healthy plants followed by *P. niruri* pre-treated plants. In post-inoculation studies, biomass of the treated plants was less compared to the pre-inoculated plants and it was the highest in *P. indica*-colonized plants than other treatments (Table 10). Similar to *P. indica*, Pi-WDE treated plants also increased root and shoot biomass of the plant and significantly reduced the infection of BlCMV as evidenced by low vulnerability index in the treated plants compared to the virus treated plants.

4.6. PHYSICAL AND CHEMICAL PROPERTIES OF AVPs

Plants having potential antiviral principles were evaluated against BlCMV infection in *C. amaranticolor*. It was found that *P. niruri* and *B. diffusa* were identified as the most effective AVPs against BlCMV at 1 per cent concentration. Pre-treatment of plants with the above AVPs prior to inoculation with virus have remarkable reduction in virus compared to post-inoculation. Hence, these two AVPs at one per cent concentration as pre-treatment were used to analyse the physical and chemical properties like heat stability, photolysis, chemical nature and molecular size in local lesion host, *C. amaranticolor*, against BlCMV.

Table 10. Effect AVPs of *P. niruri* and *B. diffusa*, *P. indica*-priming and Pi-WDE against BlCMV in vegetable cowpea

Treatments	Method of application	Vulnerability index at different intervals of BlCMV inoculation in vegetable cowpea				Days for symptom appearance	Shoot biomass (g)	Root biomass (g)
		5 DAI	10 DAI	15 DAI	30 DAI			
T_1 (Control)		0	0	0	0	0	124.37±2.01	18.31±2.08
T_2 (BlCMV alone)		20.00	46.66	66.66	73.33	7	13.65±2.43	4.65±1.98
T_3 (*P. niruri* + BlCMV)	Pre-inoculation	13.33	20.00	33.33	33.33	8	113.03±2.05	13.79±3.37
T_4 (*B. diffusa* + BlCMV)		6.00	20.00	40.00	46.66	8	39.09±5.9	6.71±1.41
T_5 (*P. indica* + BlCMV)		10.00	13.33	20.00	20.00	8	144.89±3.28	22.7±2.4
T_6 (Pi-WDE + BlCMV		6.0	26.66	33.33	40.00	7	72.67±13.53	17.18±1.94
T_7 (BlCMV + *P. niruri*)	Post-inoculation	13.33	26.66	33.33	40.00	8	24.59±0.95	2.79±0.9
T_8 (BlCMV + *B. diffusa*)		13.33	26.66	40.00	53.33	8	17.78±3.02	2.25±0.45
T_9 (BlCMV + *P. indica*)		0.0	20.00	26.66	46.66	8	28.45±1.96	6.27±1.63
T_{10} (BlCMV + Pi-WDE)		10.00	26.66	33.33	46.66	7	24.51±4.2	6.7±2.89
T_{11} (*P. indica* alone)		0	0	0	0	0	128.68±14.48	28.45±5.8
SE(m) ±							6.63	2.64
CD (0.05)							19.46	7.75

- Values are the mean of three replications ± standard deviation

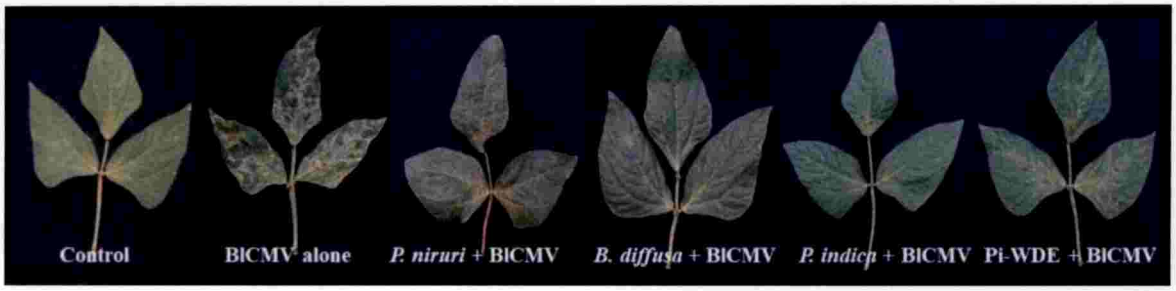


Plate 24. Vulnerability index of pre-treated cowpea leaves with AVPs of *P. niruri* and *B. diffusa*, *P. indica* and its WDE against BlCMV after 30 days of treatment.

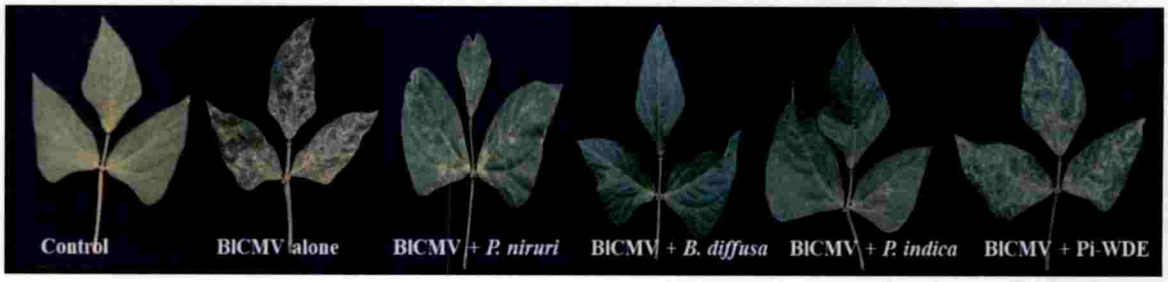


Plate 25. Vulnerability index of post-treated cowpea leaves with AVPs *of P. niruri* and *B. diffusa* , *P. indica* and its WDE against BlCMV after 30 days of treatment.

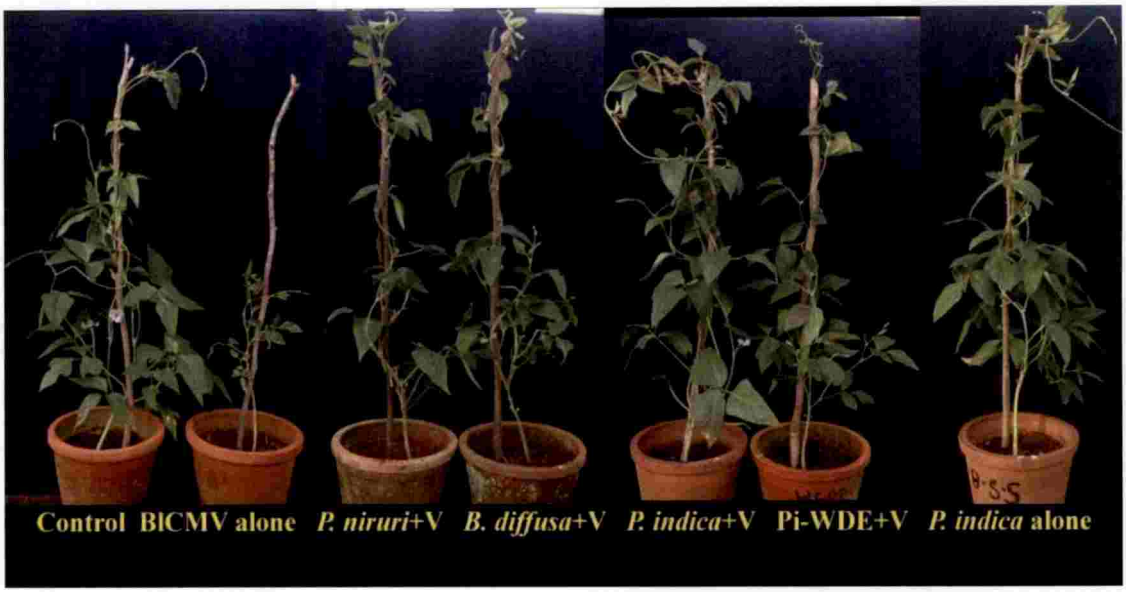


Plate 26. Pre treatment of cowpea with AVPs of *P. niruri* and *B. diffusa*, *P. indica* and its WDE against BlCMV after 30 days of treatment.

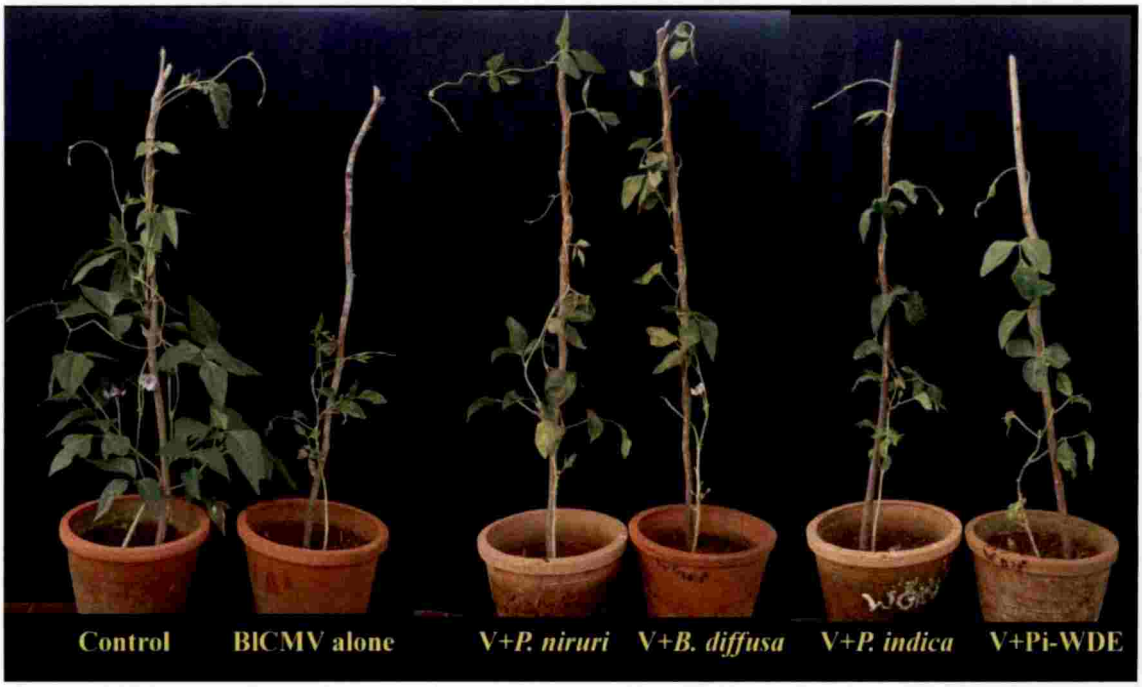


Plate 27. Post treatment of cowpea with AVPs of *P. niruri* and *B. diffusa*, *P. indica* and its WDE against BlCMV after 30 days of treatment.

4.6.1. Thermostability of AVPs of *P. niruri* and *B. diffusa*

Aqueous extracts of the AVPs were subjected to moist heat of 121°C for 20 minutes (Autoclaved) to evaluate the thermostability of these extracts. It was found that autoclaved plant extracts of both the AVPs reduced the BlCMV local lesions in *C. amaranticolor* than that of non-autoclaved AVPs extracts (Table 11, Plate 28). The above results indicated that the AVPs from *P. niruri* and *B. diffusa* were thermostable at 121°C for 20 min.

4.6.2. Photolysis of AVPs of *P. niruri* and *B. diffusa*

The AVPs from *P. niruri* and *B. diffusa* exposed to open sunlight were used to study its photolysis property. *P. niruri* extracts exposed to light from 8 AM TO 5 PM inhibited / reduced 53 per cent of local lesions over control whereas light non-exposed treatment inhibits 82 per cent of local lesions by BlCMV (Table 12; Plate 29). *B. diffusa* extracts exposed to light also inhibited the local lesions to 64 per cent whereas non-exposed inhibit 76 per cent over control (Table 12; Plate 29). The results revealed that the plants treated with light exposed extracts produced more number of lesions than light non-exposed extracts of both the AVPs and thereby confirming both the AVPs as partially photosensitive.

4.6.3. Chemical nature of AVPs (protein/non-protein)

Protein and non-protein fractions of AVPs obtained after methanol extraction were applied to plants to determine the active antiviral principles against BlCMV in *C. amaranticolor*. Protein fraction of *P. niruri* significantly reduced / inhibited the local lesions which was similar to that of aqueous extracts but the local lesion appeared on non-protein part treated leaf was similar as that of the virus alone inoculated leaf (Table 13, Plate 30). Similar results were obtained with protein fraction from *B. diffusa* which could inhibit 83 per cent of local lesions over control, whereas non-protein fraction did not reduce the number of local lesions formed (Table 13, Plate 30). The result revealed that active antiviral principle is protein in both *P. niruri* and *B. diffusa* and non-protein part have no significant role in reduction of local lesion developed by BlCMV.

Table 11. Effect of autoclaved AVPs of *P. niruri* and *B. diffusa* against BlCMV in local lesion host, *C. amaranticolor*

Antiviral principles	Treatments	Days for symptom appearance	Size of lesions (mm)	Number of lesions	Per cent of inhibition over control
Phyllanthus niruri	Non-autoclaved	5	1.5	5.33±0.33	82.16±2.94
	Autoclaved	5	1.5	4.33±0.33	81.60±1.38
Boerhavia diffusa	Non-Autoclaved	5	1.5	6.6±0.33	79.30±2.13
	Autoclaved	5	1.5	6.0±0.57	77.01±0.38
Control		5	1.5	29.0±2.3	00.00
SE(m) ±				1.09	1.4
CD (0.05)				3.4	3.7

- Values are the mean of three replications ± standard deviation

Table 12. Effect of light exposed AVPs of *P. niruri* and *B. diffusa* against BlCMV in local lesion host, *C. amaranticolor*

Antiviral principles	Treatments	Days for symptom appearance	Size of lesion (mm)	Number of lesion	Per cent of inhibition over control
Phyllanthus niruri	Light non-exposed	5	1.5	5.33±0.88	82.16±2.94
	Light exposed	5	1.5	14±1.52	53.33±5.22
Boerhavia diffusa	Light non-exposed	5	1.5	7±0.57	76.3±2.02
	Light exposed	5	2.0	10.67±0.33	64.4±1.1
Control		5	1.5	30.33±2.18	00.00
SE(m) ±				1.29	3.21
CD (0.05)				4.06	10.47

- Values are the mean of three replications ± standard deviation

Table 13. Effect of the protein and non-protein fractions of AVPs of *P. niruri* and *B. diffusa* against BlCMV in local lesion host, *C. amaranticolor*

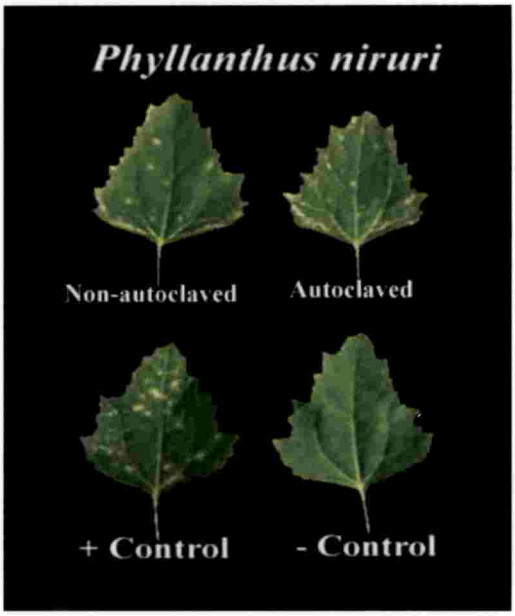
Antiviral principles	Treatments	Days for symptom appearance	Size of lesion (mm)	Number of lesion	Per cent of inhibition over control
Phyllanthus niruri	Crude	5	2.0	5.33±0.33	81.60±1.14
	Protein	5	2.0	6.33±0.88	78.15±3.04
	Non-protein	5	2.0	24.0±0.57	17.27±1.98
Boerhavia diffusa	Crude	5	2.0	6.67±0.33	77.01±1.15
	Protein	5	2.0	4.66±0.88	83.93±3.04
	Non-protein	5	2.0	22.33±0.66	22.98±2.30
Control		5	2.0	29.00±2.30	
SE(m) ±				1.06	2.25
CD (0.05)				3.25	6.0

- Values are the mean of three replications ± standard deviation

Table 14. Effect of the different molecular size sieved AVPs of *P. niruri* and *B. diffusa* against BlCMV in local lesion host, *C. amaranticolor*

Antiviral principles	Molecular size	Days for symptom appearance	Size of lesion (mm)	Number of lesion	Per cent of inhibition over control
Phyllanthus niruri	>10 kDa	5	1.5	3.33±0.57	87.64±2.13
	10-1 kDa	5	1.5	5.33±1.52	80.24±5.65
	<1 kDa	5	1.5	24.00±1	13.57±2.13
Boerhavia diffusa	>10 kDa	5	1.5	5.0±1	80.24±4.27
	10-1 kDa	5	1.5	5.0±1.7	76.53±4.28
	<1 kDa	5	1.5	22.33±1.54	17.28±4.28
Control		5	1.5	28.66±1.52	
SE(m) ±					2.13
CD (0.05)					6.66

- Values are the mean of three replications ± standard deviation



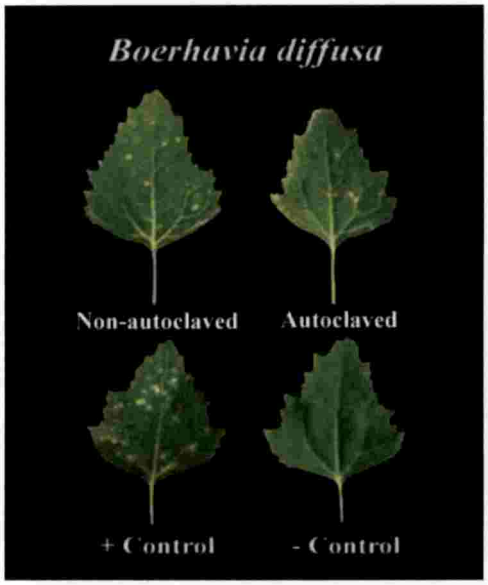
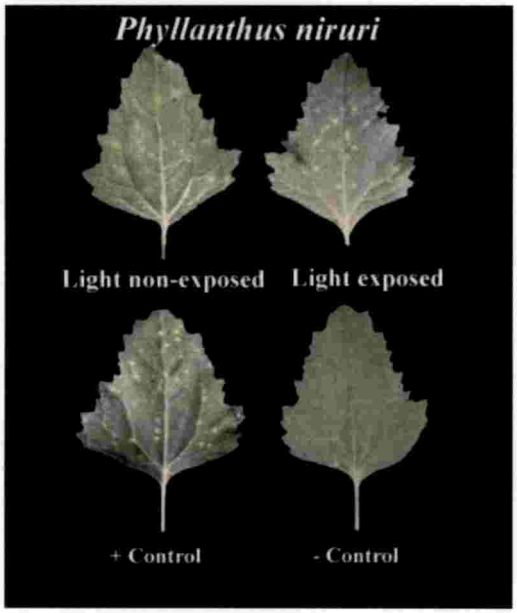


Plate 28 . Effect of moist heat on antiviral principles of *P. niruri* and *B. diffusa* on BlCMV in local lesion host, *C. amaranticolor*



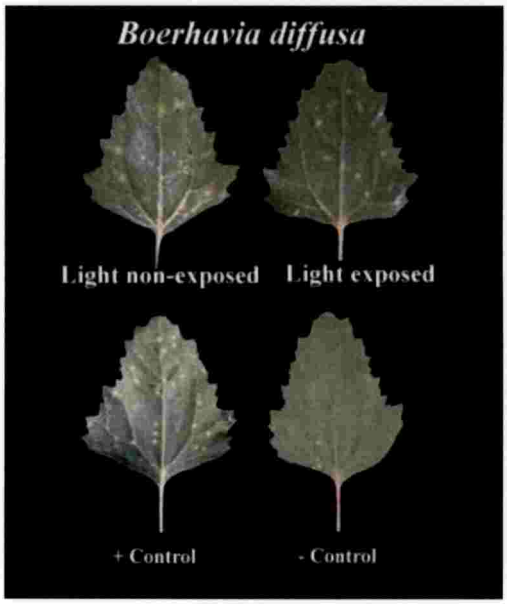


Plate 29. Effect of light exposure on antiviral principles of *P. niruri* and *B. diffusa* on BlCMV in local lesion host, *C. amaranticolor*.

4.6.4. Molecular size of anti-viral protein(s) of *P. niruri* and *B. diffusa*

The proteins present in the aqueous extracts of AVPs were precipitated and sieved depending on the molecular size. AVPs having molecular size of more than 10 kDa and 1-10 kDa from both *P. niruri* and *B. diffusa* significantly inhibited more than 80 and 75 per cent of local lesions produced by BlCMV respectively, whereas the per cent inhibition was the least in molecular size of less than 1 kDa (Table 14, Plate 31). The above result indicated that the molecular size of anti-viral proteins from both the AVPs were more than 1 kDa.

4.7. BIOCHEMICAL CHANGES OF HOST PATHOGEN INTERACTIONS

The biochemical mechanisms involved in the resistance or tolerance of *P. indica*-primed plants, plants treated with AVPs and Pi-WDE were studied. The biochemical parameters were total soluble protein, total phenol, defense enzymes and induction of PR-proteins.

4.7.1. Estimation of total soluble protein

Total soluble proteins in treated plants were carried out as per the procedure given by Bradford (1976) and the results are presented in Table 15. The results revealed that protein content in treated, virus inoculated and healthy plants were increased and reached a maximum value at 15 DAI. There after protein content declined at 30 DAI. *P. indica*-primed plants with BlCMV inoculated reached maximum protein content at 10 DAI (3.10 mg g^{-1}) and maintained same level at 15 DAI, there after it declined at 30 DAI (2.08 mg g^{-1}). There was no significant difference in protein content among the treated plants with the tolerance or resistance to blackeye cowpea mosaic disease.

4.7.2. Estimation of phenol

Inoculation of virus to the *P. indica*-primed, AVP and Pi-WDE treated plants along with healthy and virus inoculated plants recorded an increase in phenol content and maximum phenol content was recorded at 30 DAI. AVP treated plants increased several fold of phenol content compared to other treatments including the healthy and the virus inoculated plants. The phenol

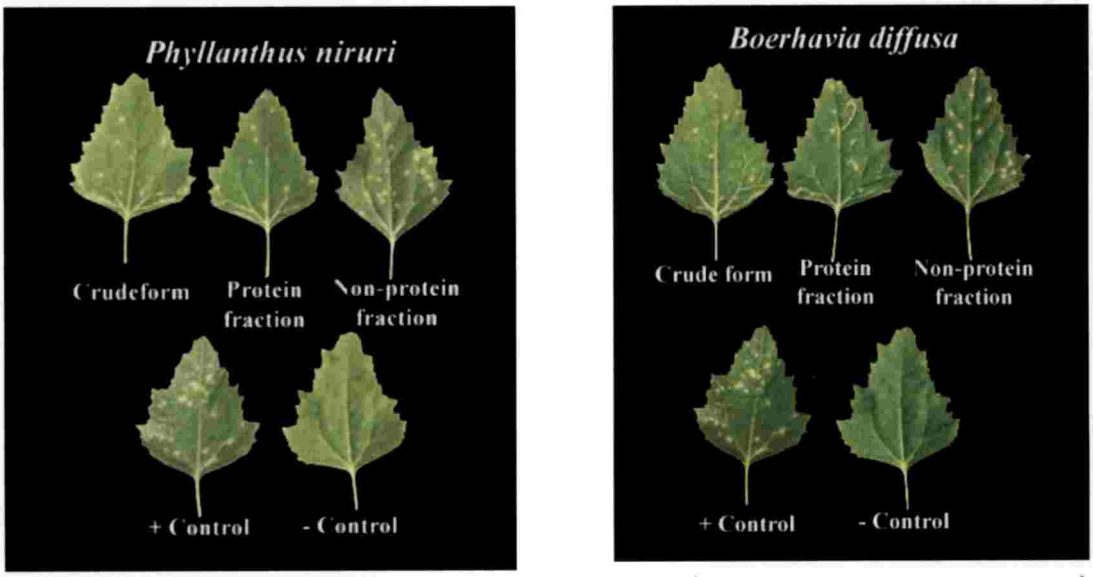


Plate 30. Effect of protein and non-protein fractions of antiviral principles of *P. niruri* and *B. diffusa* on BlCMV in local lesion host, *C. amaranticolor.*

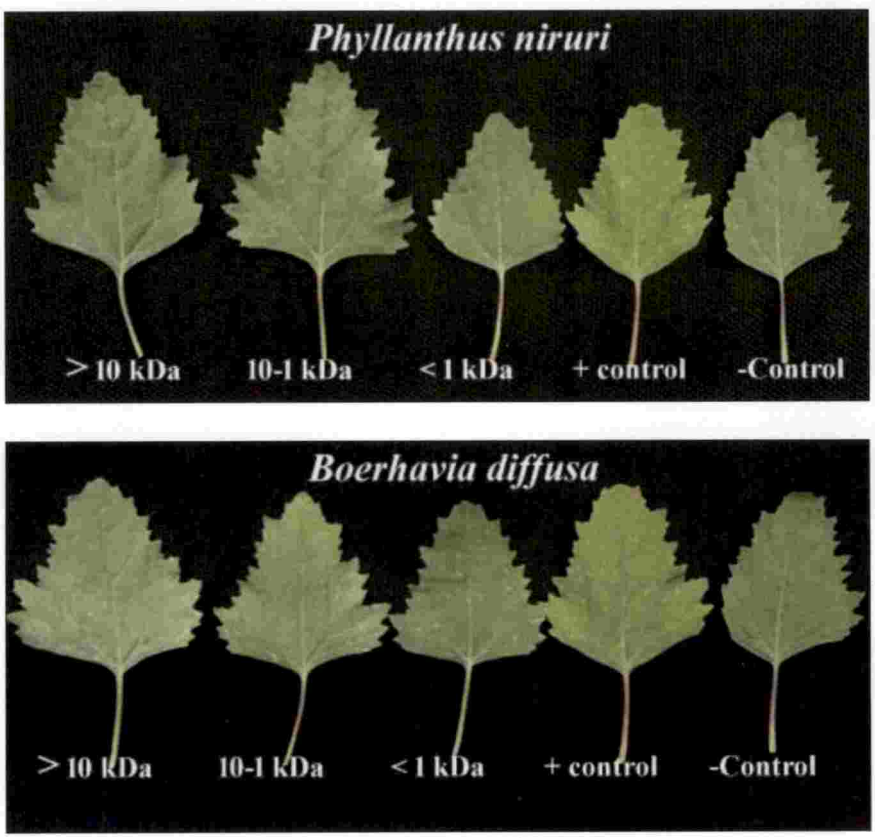


Plate 31. Effect of different size of antiviral proteins from *P. niruri* and *B. diffusa* on BlCMV in local lesion host, *C. amaranticolor.*

Table 15. Changes in total soluble protein content of cowpea leaves treated with AVPs of *P. niruri* and *B. diffusa*, *P. indica*-priming and Pi-WDE against BlCMV

Treatments	Total soluble proteins content (mg g^{-1} fresh weight of tissue)			
	5 DAI	10 DAI	15 DAI	30 DAI
Healthy	2.23±0.12	1.82±0.05	3.82±0.08	1.89±0.06
BlCMV alone	2.26±0.09	1.76±0.06	3.29±0.05	1.69±0.04
P. niruri + BlCMV	2.26±0.09	1.87±0.03	2.46±0.03	2.28±0.02
B. diffusa + BlCMV	2.38±0.03	1.56±0.08	2.45±0.03	1.98±0.03
P. indica + BlCMV	2.16±0.04	3.10±0.09	3.09±0.06	2.08±0.02
Pi-WDE + BlCMV	2±0.02	2.52±0.09	3.01±0.03	1.78±0.04
P. indica alone	2.25±0.08	2.98±0.01	2.62±0.02	2.46±0.05
SE(m) ±	0.08	0.06	0.05	0.03
CD(0.05)		0.2	0.15	0.11

- DAI – Days after inoculation
- Values are the mean of three replications ± standard deviation

Table 16. Changes in total phenol content of cowpea leaves treated with AVPs of *P. niruri* and *B. diffusa*, *P. indica*-priming and Pi-WDE against BlCMV

Treatments	Phenol content (mg g^{-1} fresh weight of tissue)			
	5 DAI	10 DAI	15 DAI	30 DAI
Healthy	1.53±0.06	1.66±0.03	2.73±0.13	4.93±0.06
BlCMV alone	0.93±0.06	2.53±0.03	3.86±0.13	6.2±0.11
P. niruri + BlCMV	3.53±0.17	4.93±0.12	3.86±0.13	5.4±0.11
B. diffusa + BlCMV	2.54±0.05	3.3±0.05	2.13±0.04	3.53±0.03
P. indica + BlCMV	0.73±0.06	2.73±0.08	2.13±0.06	6.8±0.11
Pi-WDE + BlCMV	1.26±0.06	2.33±0.06	3.53±0.06	6.06±0.13
P. indica alone	1.00±0.11	2.16±0.03	4.2±0.06	4.73±0.06
SE(m) ±	0.03	0.07	0.07	0.09
CD (0.05)	0.41	0.23	0.3	0.3

- DAI – Days after inoculation
- Values are the mean of three replications ± standard deviation

content of *P. niruri* treated plants increased several fold over healthy, virus inoculated and other treated plants upto10 DAI (4.93 mg g^{-1}) (Table 16). Similar to *P. niruri*, *B. diffusa* treated plants initially increased the phenol content over healthy and virus inoculated plants, but at 30 DAI, phenol content was low among the treatments including the healthy and virus inoculated plants (Table 16). *P. indica*-primed and Pi-WDE treated plants found similar trend in phenol content with healthy and virus inoculated plants (Table 16).

4.7.3. Estimation of Peroxidase

The changes in the enzyme activities in the treated plants were summarized in Table 17. Peroxidase (PO) activity was increased in all the virus inoculated plants over healthy. PO activity in *P. indica*-primed (81.37 mg g^{-1} min^{-1}) and *P. niruri* treated (53.4 mg g^{-1} min^{-1}) plants was higher compared to healthy and virus inoculated plants at 10 DAI. It was found that the PO activity was increased throughout the period of study except for Pi-WDE and *B. diffusa* treated plants where it started declining from 10 DAI and 15 DAI onwards respectively.

4.7.4. Estimation of Polyphenol oxidase

The activity of enzyme polyphenol oxidase in treated plants with healthy and virus inoculated plants presented in Table 18. The enzyme activity in *P. niruri* treated plants increased throughout the study (3.72 mg g^{-1} min^{-1}). PPO activity reached maximum in *P. indica*-primed (3.44 mg g^{-1} min^{-1}) and *B. diffusa* treated (2.69 mg g^{-1} min^{-1}) plants at 10 DAI and thereafter gradually declined. The higher PPO activity in Pi-WDE treated plants was recorded at 15 DAI and thereafter rapidly decreased at 30 DAI.

4.7.5. Estimation of Phenylalanine ammonialyase

Phenylalanine ammonialyase activity in AVPs treated, healthy and virus inoculated plants decreased gradually with the time period but the *P. indica*-primed with virus inoculation recorded initial increase at 10 DAI, thereafter the activity of PAL decreased till 30 DAI. Continuous increase in PAL activity was

Table 17. Changes in Peroxidase activity of cowpea leaves treated with AVPs of *P. niruri* and *B. diffusa*, *P. indica*-priming and Pi-WDE against BlCMV

Treatments	Peroxidase activity (mg g^{-1} min^{-1} fresh weight of tissue)			
	5 DAI	10 DAI	15 DAI	30 DAI
Healthy	20.75±0.14	35.25±0.1	54.31±0.07	78.92±0.01
BlCMV alone	37.36±0.08	41.41±0.04	63.76±0.02	56.72±0.07
P. niruri + BlCMV	41.25±0.14	53.40±0.08	78.26±0.02	84.34±0.02
B. diffusa + BlCMV	36.85±0.06	47.74±0.08	58.55±0.06	50.19±0.01
P. indica + BlCMV	51.25±0.02	81.37±0.04	71.57±0.24	79.44±0.01
Pi-WDE + BlCMV	66.66±0.09	87.42±0.1	74.40±0.05	56.18±0.02
P. indica alone	25.91±0.04	34.28±0.06	58.23±0.03	68.40±0.03
SE(m) ±	0.17	0.13	0.1	0.04
CD(0.05)	0.54	0.42	0.3	0.13

- DAI – Days after inoculation
- Values are the mean of three replications ± standard deviation

Table 18. Changes in Polyphenol oxidase activity of cowpea treated with AVPs of *P. niruri* and *B. diffusa*, *P. indica*-priming and Pi-WDE against BlCMV

Treatments	Polyphenol oxidase activity (mg g^{-1} min^{-1} fresh weight of tissue)			
	5 DAI	10 DAI	15 DAI	30 DAI
Healthy	0.92±0.05	2.34±0.02	4.33±0.02	3.5±0.04
BlCMV alone	1.05±0.03	5.22±0.01	2.25±0.02	2.32±0.02
P. niruri + BlCMV	1.26±0.29	2.36±0.01	3.70±0.07	3.72±0.02
B. diffusa + BlCMV	0.78±0.01	2.69±0.01	2.44±0.02	2.73±0.02
P. indica + BlCMV	1.24±0.01	3.44±0.01	3.22±0.01	2.53±0.04
Pi-WDE + BlCMV	0.83±0.02	1.55±0.01	3.13±0.08	1.74±0.06
P. indica alone	0.55±0.01	0.82±0.04	2.32±0.02	3.16±0.02
SE(m) ±	0.02	0.09	0.01	0.04
CD(0.05)	0.06	0.3	0.05	0.12

- DAI – Days after inoculation
- Values are the mean of three replications ± standard deviation

Table 19. Changes in Phenylalanine ammonialyase activity of cowpea leaves treated with AVPs of *P. niruri* and *B. diffusa*, *P. indica*-priming and Pi-WDE against BlCMV

Treatments	Phenylalanine ammonialyase activity (mg g^{-1} min^{-1} fresh weight of tissue)			
	5 DAI	10 DAI	15 DAI	30 DAI
Healthy	37.50±0.72	34.58±0.83	31.25±0.72	11.00±0.75
BlCMV alone	37.29±1.77	37.08±1.1	21.41±0.58	18.75±0.72
P. niruri + BlCMV	35.0±0.72	29.58±1.1	27.50±0.72	22.91±0.83
B. diffusa + BlCMV	36.94±0.36	30.83±0.41	22.88±0.81	17.83±1.02
P. indica + BlCMV	33.05±0.36	42.11±1.07	21.66±0.41	21.25±0.72
Pi-WDE + BlCMV	23.75±0.72	24.58±0.41	33.75±0.72	35.41±0.41
P. indica alone	28.05±0.36	7.2±0.77	54.16±0.33	17.75±0.52
SE(m) ±	0.85	8.49	1.4	0.73
CD(0.05)	2.62	2.77	4.3	2.25

- DAI – Days after inoculation
- Values are the mean of three replications ± standard deviation

observed in Pi-WDE treated virus inoculated plants (35.41 mg g^{-1} min^{-1}) throughout the study (Table 19).

4.7.6. Electrophoretic analysis of PR- Proteins through SDS-PAGE

PR-proteins profiling with SDS-PAGE was done in cowpea against BlCMV treated with AVPs, *P. indica*-primed and Pi-WDE at different intervals viz., 5, 10, 15 and 30 DAI. Many proteins were induced in response to the different treatments. Induction of PR-proteins was more in *P. indica*-primed plants with and without virus inoculation at 5 DAI (Plate 32). The intensity of band having molecular weight 29.4 kDa was high in all the treatments except the virus inoculated plants. Induction of 49.4 kDa was observed in pre-treated *P. indica* and *P. niruri*. An extra band of 24.8 kDa was observed in *P. indica*-colonized virus inoculated plants.

At 10 DAI, new proteins of 30.9 kDa and 64.3 kDa were induced in *P. indica*-colonized virus inoculated plants (Plate 33). *P. niruri* treated plants produced protein with molecular weight of 34 kDa. On 15 DAI, an induced protein of 66.8 kDa was observed in all the treated plants except the healthy and virus inoculated plants (Plate 34). Protein of 18.3 kDa was observed in *P. indica*-primed plants inoculated with the virus and protein of 34 kDa was found in *P. niruri* treated plants.

No new proteins were appeared in different treatments except for *P. indica* colonized plants, which induced protein of 31.9 kDa after 30 DAI (Plate 35). *P. niruri* constantly maintained the 34 kDa protein in treated plants. A decrease in the intensity of PR-protein with time interval was observed. In BlCMV treated plants, none of the PR-proteins were induced at different intervals of the experiment.

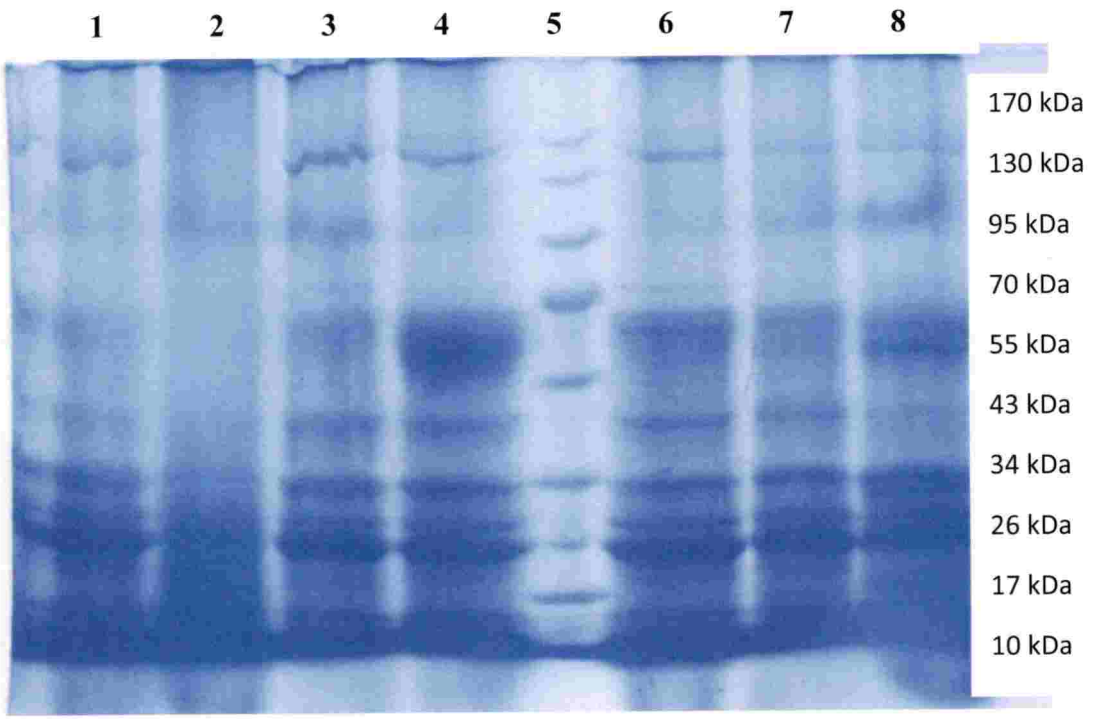


Plate 32. PR proteins profile in cowpea leaves inoculated with BlCMV and treated with AVPs *of P. niruri* and *B. diffusa* , *P. indica* and its WDE at 5 DAI.

1 – Healthy
2 – BlCMV alone
3 – *P. niruri* +BlCMV
4 – *B. diffusa* + BlCMV
5 – Marker
6 – *P. indica* + BlCMV
7 – Pi-WDE
8 – *P. indica* alone

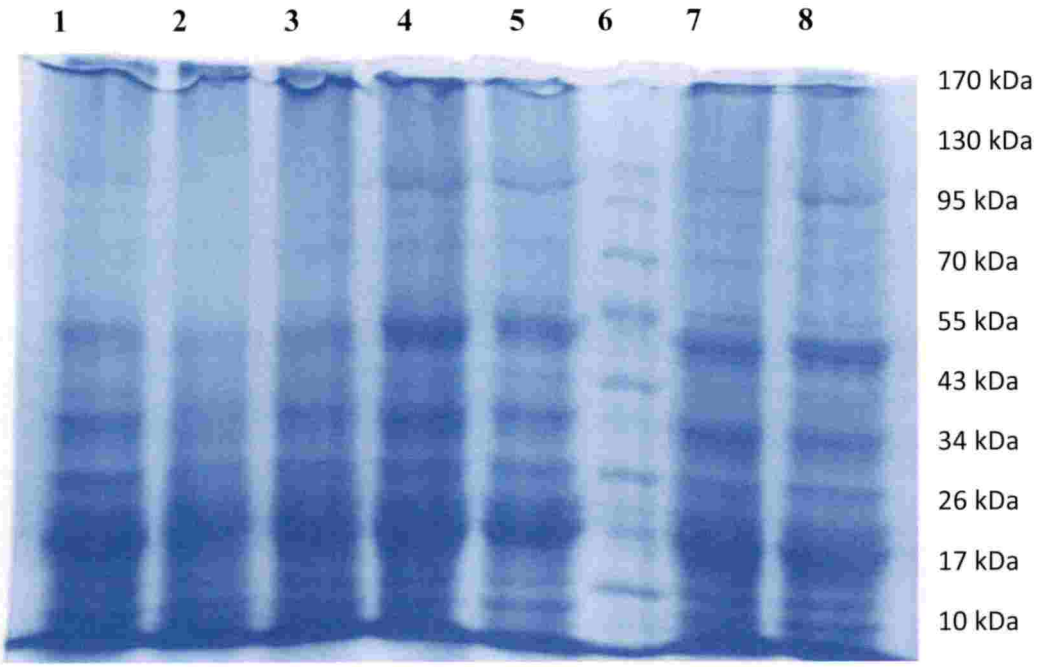


Plate 33. PR proteins profile in cowpea leaves inoculated with BlCMV and treated with AVPs *of P. niruri* and *B. diffusa* , *P. indica* and its WDE at 10 DAI.

1 – Healthy
2 – BlCMV alone
3 – *P. niruri* +BlCMV
4 – *B. diffusa* + BlCMV
5 – *P. indica* + BlCMV
6 – Marker
7 – Pi-WDE
8 – *P. indica* alone

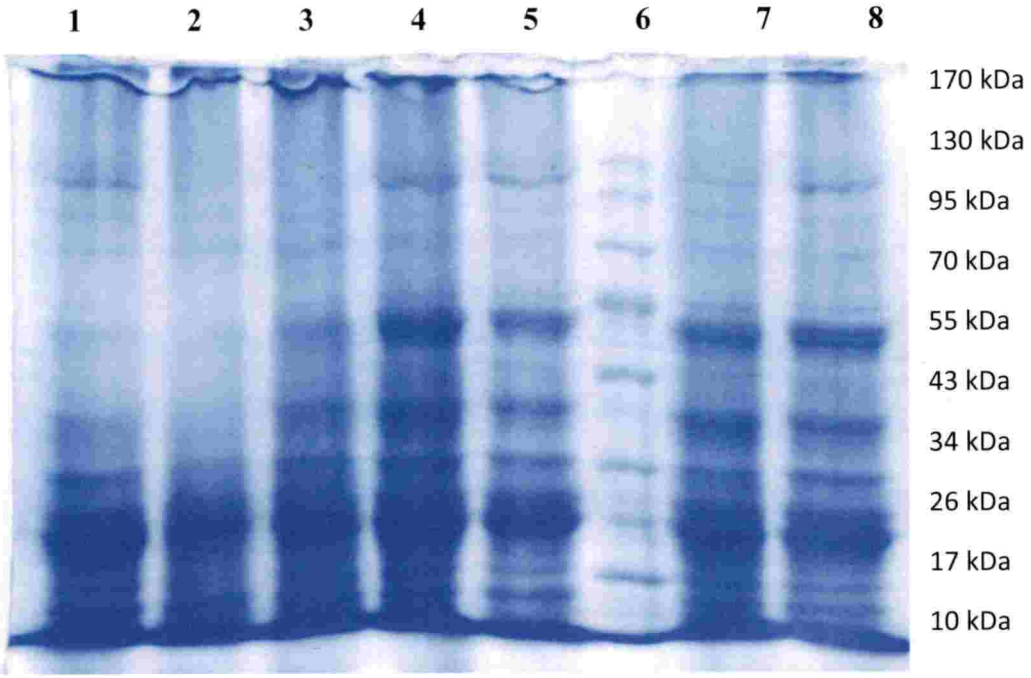


Plate 34. PR proteins profile in cowpea leaves inoculated with BlCMV and treated with AVPs *of P. niruri* and *B. diffusa* , *P. indica* and its WDE at 15 DAI.

1 – Healthy
2 – BlCMV alone
3 – *P. niruri* +BlCMV
4 – *B. diffusa* + BlCMV
5 – *P. indica* + BlCMV
6 – Marker
7 – Pi-WDE
8 – *P. indica* alone

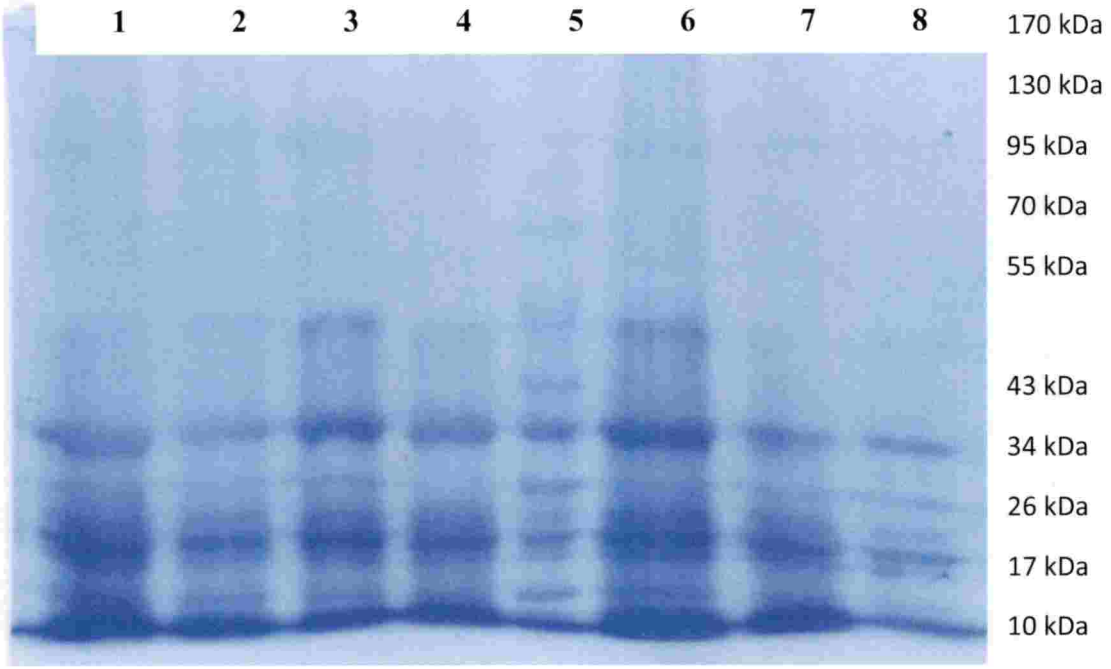


Plate 35. PR proteins profile in cowpea leaves inoculated with BlCMV and treated with AVPs *of P. niruri* and *B. diffusa* , *P. indica* and its WDE at 30 DAI.

1 – Healthy
2 – BlCMV alone
3 – *P. niruri* +BlCMV
4 – *B. diffusa* + BlCMV
5 – Marker
6 – *P. indica* + BlCMV
7 – Pi-WDE
8 – *P. indica* alone

Discussion

5. Discussion

Cowpea is a multipurpose leguminous crop, cultivated in tropical and sub-tropical regions. Potential yield of the crop is reduced by several biotic and abiotic agents and among them viruses are the major ones reducing yield of the crop and may lead to crop failure in extreme cases. Symptoms produced by virus infected crop may resemble other biotic and abiotic stress, hence it is difficult to identify. Management of viral disease is always cumbersome and one of the best options is by induction of host resistance which is the cheapest method to manage any disease. The results of the study on symptomatology, immunological detection and management of *Blackeye cowpea mosaic virus* by inducing resistance with biotic agents *viz.,* plant extracts and the fungal root endophytes, *P. indica* and its WDE are discussed in this chapter. Physical and chemical properties of the best antiviral plant extracts are also discussed in this chapter.

5.1. MAINTENANCE OF THE VIRUS AND ENDOPHYTE

5.1.1. Maintenance of virus

Blackeye cowpea mosaic virus was maintained in systemic host, vegetable cowpea (var. Sharika) and local lesion host, *C. amaranticolor* by repeated mechanical transmission using 0.1 M sodium phosphate buffer for the virus extraction. Wall *et al.* (1996) used single local lesion from *C. amaranticolor* to purify the BlCMV and the virus was maintained in yard long bean, a systemic host. BlCMV was successfully transmitted by sap transmission and maintained in vegetable cowpea and *C. amaranticolor* (Radhika, 1999; Krishnapriya, 2015; Alex, 2017). Shankar *et al.* (2009) maintained the virus by inoculating in primary leaf stage of cowpea. Pure virus isolates of BlCMV obtained from research institutes were maintained in cowpea by sap transmission with phosphate buffer (Pavithra *et al.,* 2015; Ibrahim *et al.,* 2017).

5.1.2. Symptomatology

BlCMV transmitted into primary two leaf stage of cowpea seedlings produced symptoms in emerging trifoliate leaves. The virus produced symptoms

like leaf blistering, vein clearing and vein banding on 7th DAI. Later it developed into mosaic, chlorosis, downward folding of leaves (epinasty), rugosity and reduction of leaf size. In advance stages, the plants expressed symptoms like leaf crinkling, vein netting, leaf distortion, flower malformation and reduced number of flowers and pods. Infected plants produced reduced length of pods with few seeds and infected plants had shorter inter-nodal length, thus appeared as stunting. Similar symptoms of vein clearing, vein banding and mosaic symptoms were reported by Collins *et al.* (1985), in addition to blistering and malformation (Ouattara and Chambliss, 1991; Shilpashree, 2006; Alex, 2017). Symptoms such as reduced flowers and pods with malformed leaves (Krishnapriya, 2015); upward and downward leaf curling, crinkling and mottling (Dhanasekar and Reddy, 2015) were also reported.

Vein banding and dark green islands of mosaic symptoms were noticed by Saric (1991) at 10 to 14 DAI. Additionlly blistering, leaf distortion and reduced plant height were reported (Radhika, 1999). Hao *et al.* (2003) described different symptoms of BlCMV in cowpea including downward curling, mosaic, vein clearing, vein banding, leaf malformation, chlorosis and stunting of plants which were similar to the symptom observed in the present study.

Contradictory to the present observations, Collins *et al.* (1985) had reported local necrotic lesions which appeared 14 days after mechanical inoculation of BlCMV (Collins *et al.*, 1985; Ouattara and Chambliss, 1991).

In *C. amaranticolor*, yellow chlorotic local lesions appeared five days after BlCMV inoculation which later changed into necrotic lesions. Similar observation on chlorotic local lesions turning to necrotic was reported by Pavithra *et al.* (2015). BlCMV infection was studied in *C. amaranticolor* and the virus produced localized yellow chlorotic lesions (Saric, 1991; Radhika, 1999; Veena, 2007; Alex, 2017).

5.1.3. Maintenance of the fungus

The beneficial fungal root endophyte, *P. indica* was maintained in both PDA and PDB by continuous sub-culturing. The fungus completed radial growth of the mycelium in petri plate in nine days and 16 days for mycelial mat formation in 100 ml of the broth in 250 ml conical flask. Several researchers used PDA for the maintenance of the endophyte, *P. indica* and its active stage was maintained by sub-culturing at regular intervals (Sun *et al.*, 2010; Satheesan *et al.*, 2012; Deka and Baishya, 2015; Basu *et al.*, 2016; Alex, 2017; Anith *et al.*, 2018; Jisha *et al.*, 2018).

5.2. SEROLOGICAL DETECTION OF *Blackeye cowpea mosaic virus*

5.2.1. Direct antigen coating enzyme linked immunosorbent assay

Virus infected cowpea leaves were subjected to DAC-ELISA with poly-clonal antibodies of PVY and CABMV since BlCMV also belongs to *Potyviridae*. The serological reaction of infected samples was positive to both the polyclonal antiserums by three fold increase in OD value than the healthy samples.

In concurrence to the result obtained, Huguenot *et al.* (1993) reported that the CABMV antibody detected the BlCMV in all ELISA methods. The polyclonal antibody of CABMV detected the BlCMV and there was cross-reaction between the two antiserum (Hao *et al.*2003; Krishnapriya, 2015; Alex, 2017). Pavithra *et al.* (2015) reported that the *Bean common mosaic virus – Blackeye cowpea mosaic virus* was detected by polyclonal antibody of potyvirus.

5.2.2. Dot immunobinding assay

DIBA was performed to confirm the presence of BlCMV in collected infected leaf samples with polyclonal antiserum specific to CABMV and PVY. The infected samples reacted with both the antisera and yielded positive results to both the polyclonal antibodies by developing purple colour. The mean OD value was higher in infected samples than the healthy sample. Ali *et al.* (2017) had studied the distribution and prevalence of soybean viral diseases in Oklahoma by

DIBA. Krishnapriya (2015) and Alex (2017) also used the polyclonal antibody of CABMV to detect BlCMV in cowpea, which was similar to the present study.

5.3. STANDARDIZATION OF CO-CULTIVATION METHOD FOR *P. indica* WITH COWPEA AND *C. amaranticolor*

5.3.1. *In vitro* co-cultivation of *P. indica* with *C. amaranticolor* and cowpea

Cowpea seedlings when transferred from MS medium to PNM in 9 cm petri dishes for colonization with *P. indica* showed indication of rotting as the foliage of cowpea was too much to accommodate in the petri dish. Lin *et al.* (2019) found that *P. indica* colonized anthurium roots at 14 DAC under *in vitro*. These seedlings also decayed from 9 days by shedding the leaves. It might be due to the unfavourable conditions inside the jam bottle. Cowpea seeds were directly placed in PNM media with *P. indica,* and mycelium was observed in roots at 5 DAC, chlamydospores on the root surface at 7 DAC and chlamydospores inside the roots at 14 DAC.

Chenopodium seedling from MS medium placed in PNM medium with the fungus for co-cultivation had no further growth and development as the PNM medium did not support the growth of the plant. *C. amaranticolor* seeds were directly placed in PNM media and colonization was assessed after three weeks of co-cultivation. Chlamydospores and colonization was observed within the roots. Alex (2017) noticed similar observation with cowpea seedlings maintained *in vitro,* decaying due to the inadequate space for trailing type cowpea (var. Sharika). She also reported the failure of *P. indica* colonization in *C. amaranticolor* seedlings under *in vitro* condition which was contradictory to the present observation.

5.3.3. *In vivo* co-cultivation of *P. indica* with cowpea and *C. amaranticolor*

Cowpea and *C. amaranticolor* seedlings co-cultivated with *P. indica* were assessed for colonization. In cowpea, mycelium penetrated within the roots at 5 DAC. Fungal chlamydospores were observed on the roots at 7 DAC. Formation of chlamydospores inside the root was initiated on 10 DAC and mature

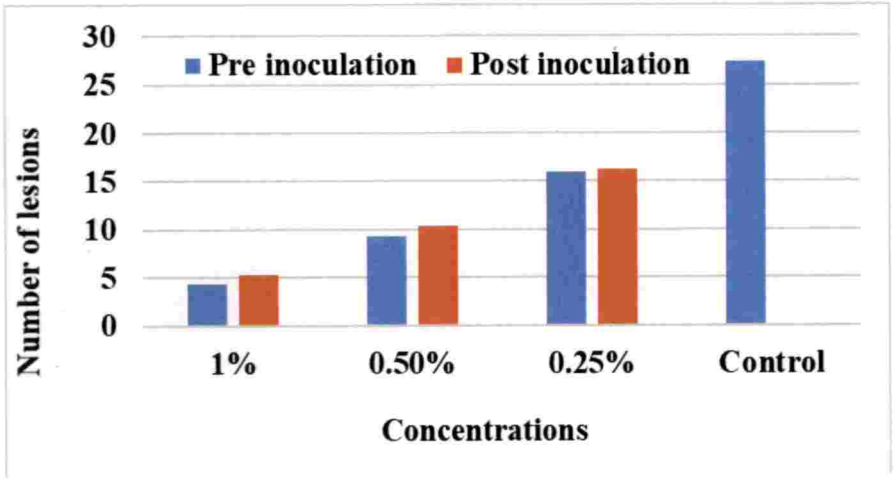


Fig. 1. Inhibition of BlCMV local lesions in *C. amaranticolor* by *P. niruri* aqueous extract.

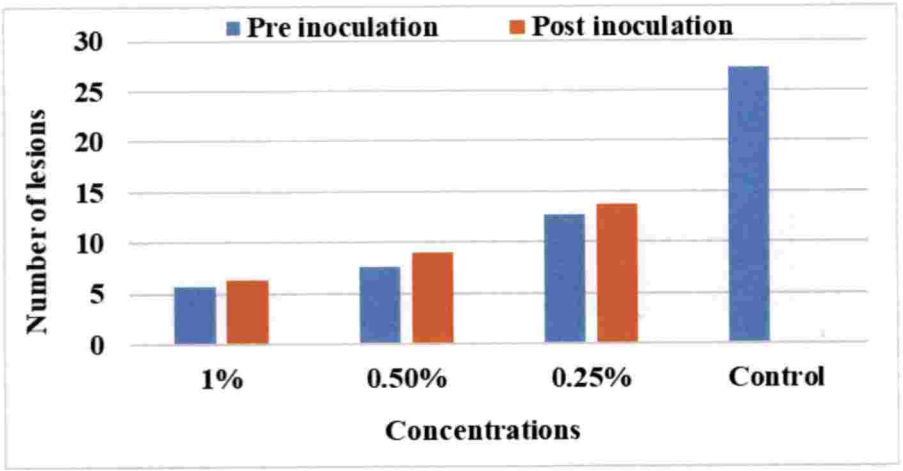


Fig. 2. Inhibition of BlCMV local lesions in *C. amaranticolor* by *B. diffusa* aqueous extract.

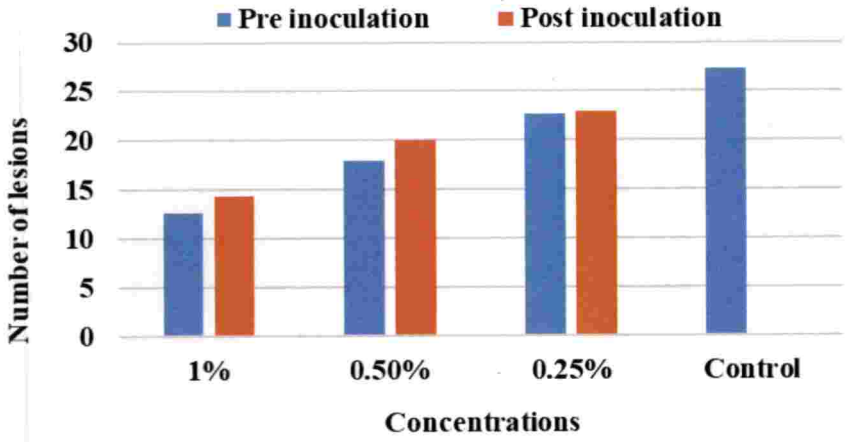


Fig. 3. Inhibition of BlCMV local lesions in *C. amaranticolor* by *D. metel* aqueous extract.

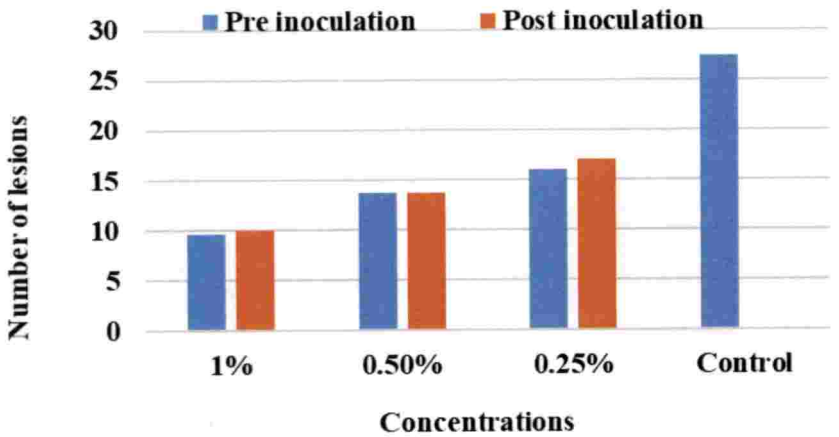


Fig. 4. Inhibition of BlCMV local lesions in *C. amaranticolor* by *P. juliflora* aqueous extract.

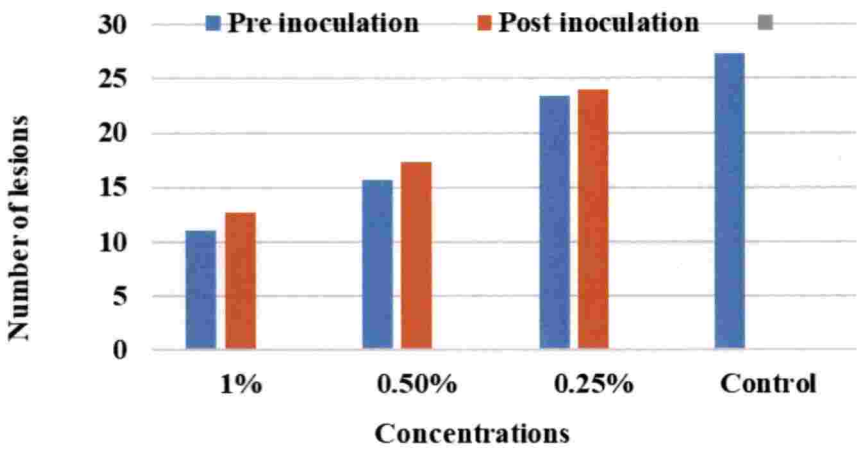


Fig. 5. Inhibition of BlCMV local lesions in *C. amaranticolor* by *P. granatum* aqueous extract.

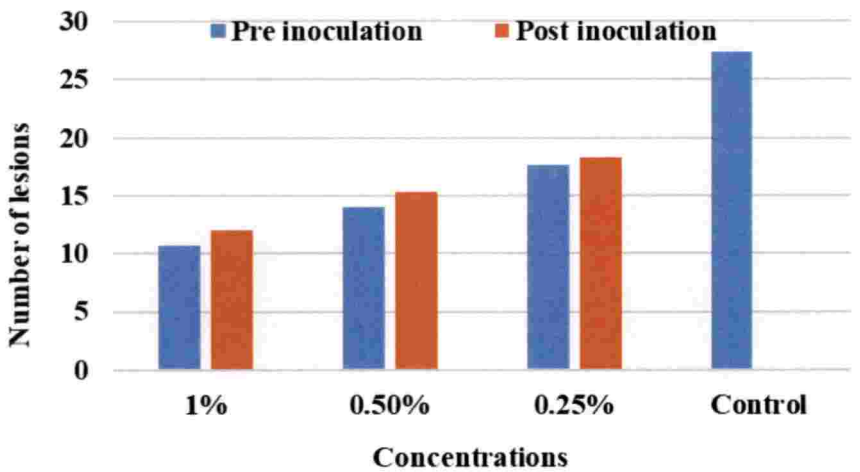


Fig. 6. Inhibition of BlCMV local lesions in *C. amaranticolor* by *E. globulus* aqueous extract.

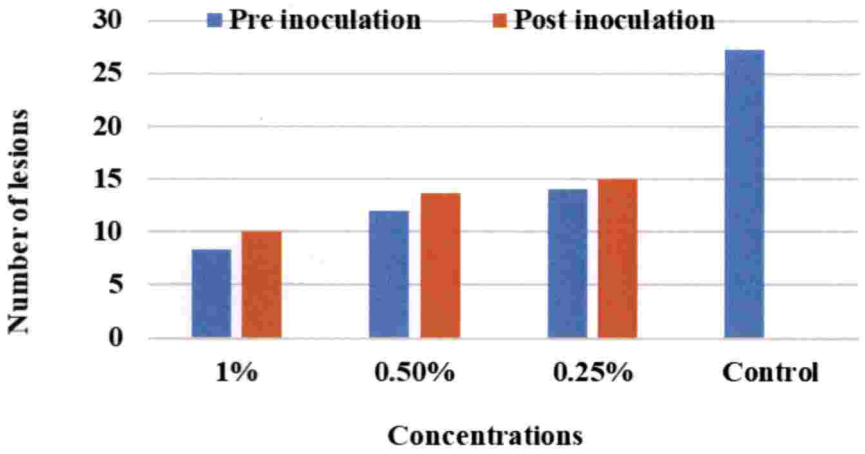


Fig. 7. Inhibition of BlCMV local lesions in *C. amaranticolor* by *C. cassia* aqueous extract.

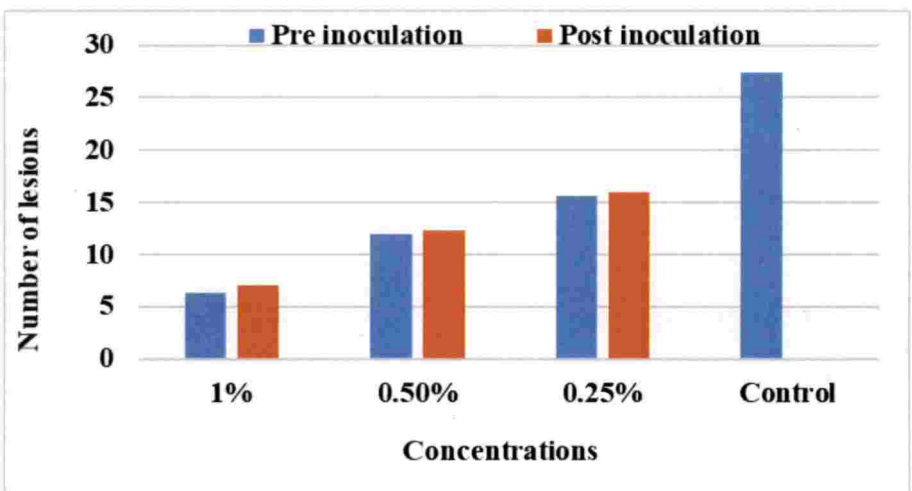


Fig. 8. Inhibition of BlCMV local lesions in *C. amaranticolor* by *P. emblica* aqueous extract.

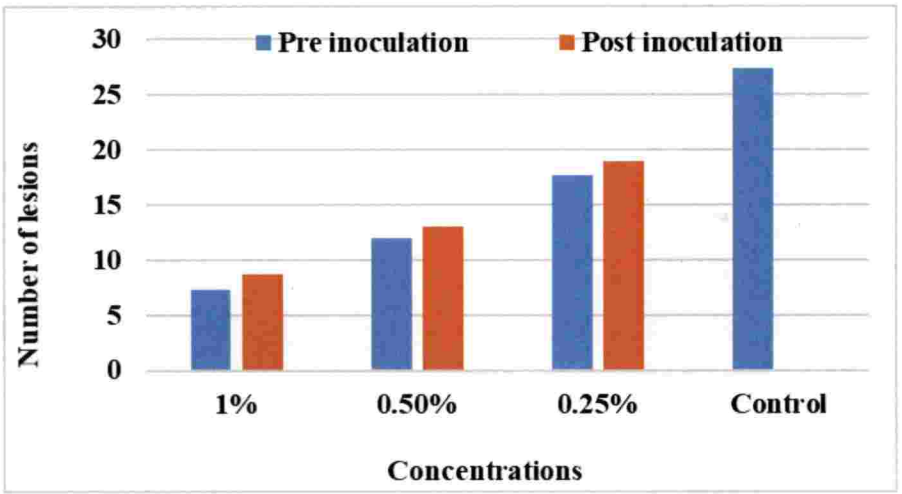


Fig. 9. Inhibition of BlCMV local lesions in *C. amaranticolor* by *M. oleifera* aqueous extract.

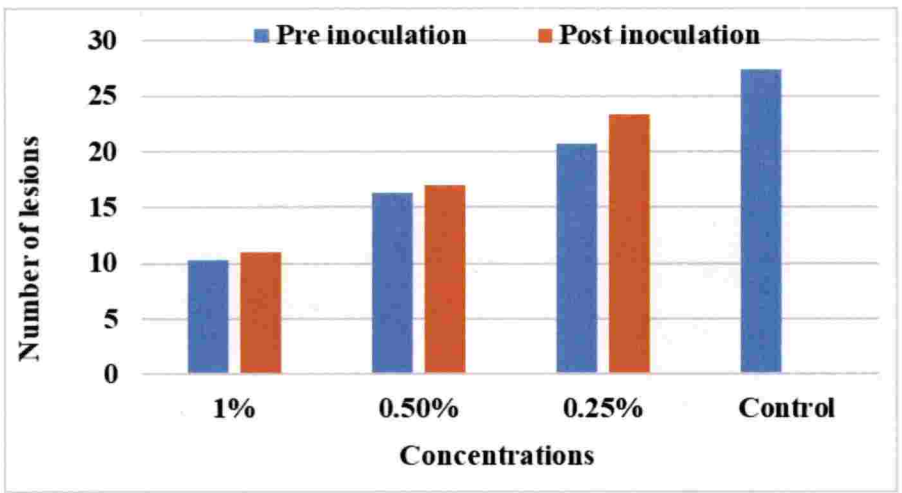


Fig. 10. Inhibition of BlCMV local lesions in *C. amaranticolor* by *V. trifolia* aqueous extract.

chlamydospores were observed at 14 DAC. Narayan *et al.* (2017) observed chlamydospores in chickpea at 5-7 DAC with *P. indica* and percentage of colonization in roots depends on culture inoculum and length of co-cultivation. Similar result of *P. indica* colonization with cowpea was reported (Alex, 2017).

In vivo co-cultivation studies with *C. amaranticolor* and *P. indica* revealed that the fungus could successfully colonize the roots at 7 DAC. The co-cultivated roots contained chlamydospores on the root surface at 14 DAC and inside the roots at 21 DAC. Contradictory to the present findings, Alex (2017) reported no colonization of *P. indica* in roots of *C. amaranticolor* under *in vivo* study.

5.4 MANAGEMENT OF BlCMV USING BOTANICALS, FUNGAL ROOT ENDOPHYTE AND ITS PRODUCT ON *C. amaranticolor*

5.4.1. Screening of aqueous plant extracts having antiviral principles on BlCMV in *C. amaranticolor*

Ten botanicals having potential AVPs were evaluated against BlCMV in local lesion host, *C. amaranticolor*. These studies revealed that the highest inhibition of local lesion was recorded in *P. niruri* sprayed plants (Fig. 1) followed by *B. diffusa* at 1 per cent (Fig. 2). In accordance with the present findings, Prasad *et al.* (2007) reported that the foliar application of *B. diffusa* at 0.75 per cent was effective in reducing the disease incidence in cowpea against BlCMV.

Virus inhibiting property of *B. diffusa* against *Bottlegourd mosaic virus* in bottlegourd (Kumar and Aswathi, 2007), *Tobacco mosaic virus* in tobacco (Lohani *et al.*, 2007), *Cowpea aphid borne mosaic virus* in cowpea (Veena, 2007), *Urdbean yellow mosaic virus* in urdbean and mungbean (Singh *et al.*, 2011), *Cucumber mosaic virus* in cucumber, *Tomato mosaic virus* and *Tomato leaf curl virus* in tomato (Najam *et al.*, 2017), *Blackeye cowpea mosaic virus* in vegetable cowpea (Alex, 2017) and watermelon viral diseases (Sharma *et al.*, 2017) had been reported.

Virus inhibiting property of *P. niruri* against *Cowpea aphid borne mosaic virus* in vegetable cowpea (Veena, 2007), *Blackeye cowpea mosaic virus* in vegetable cowpea (Alex, 2017) and scadbood virus in honeybees (Aruna *et al.*, 2017) had been reported.

Antiviral principles of plant origin were easily translocated in other plants, induced defense response and produced virus inhibitory proteins which impart resistance against plant virus (Singh *et al.*, 2011). Thuja extracts directly attached to virus particles, interfering nucleic acid translation, and thus prevented replication (Elbeshehy *et al.*, 2015). Aswathi *et al.* (2016) studied the multifaceted protection by *B. diffusa* glycoprotein against plant virus. It directly attached to degrade virus particles or coated over virus particles or assembled around the virus particles. It also induced plants to produce antiviral proteins of low molecular weight and enhanced systemic resistance in plants against virus.

Plants treated 24 h prior to virus inoculation recorded considerable level of reduction in local lesion over post treated plants. Similar findings were reported by Radhika (1999) and Vivanco *et al.* (1999). It was observed that the increase in concentration of AVPs sprayed to plants, increased the per cent inhibition of local lesions developed by BlCMV.

5.4.2. Evaluation of *P. indica*-priming in *C. amaranticolor* against BlCMV

Studies on the effect of *P. indica* root colonization in *C. amaranticolor* against BlCMV recorded 68 per cent inhibition of local lesions over control (Fig.11). The results were in agreement with previous findings that the *P. indica* colonized tomato plants which could suppress more than 75 per cent of symptoms expressed by *Tomato yellow leaf curl virus* and enhanced PAL activity with induction of PR-protein (Wang *et al.*, 2015). Fakhro *et al.* (2010) reported that *P. indica*-colonised plants were able to reduce *Pepino mosaic virus* concentration in tomato under high light intensity. Khalid *et al.* (2019) stated that *P. indica* enhanced tolerance to plants by inducing antioxidants and expressed stress regulated genes. Al-ani *et al.* (2012) found that beneficial biotic agents applied to

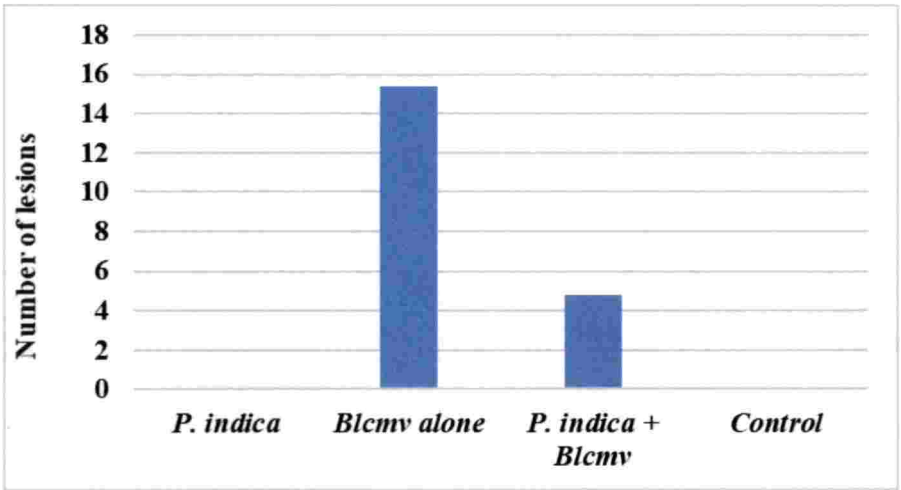


Fig. 11. Inhibition of BlCMV local lesions by *P. indica*-primed *C. amaranticolor*.

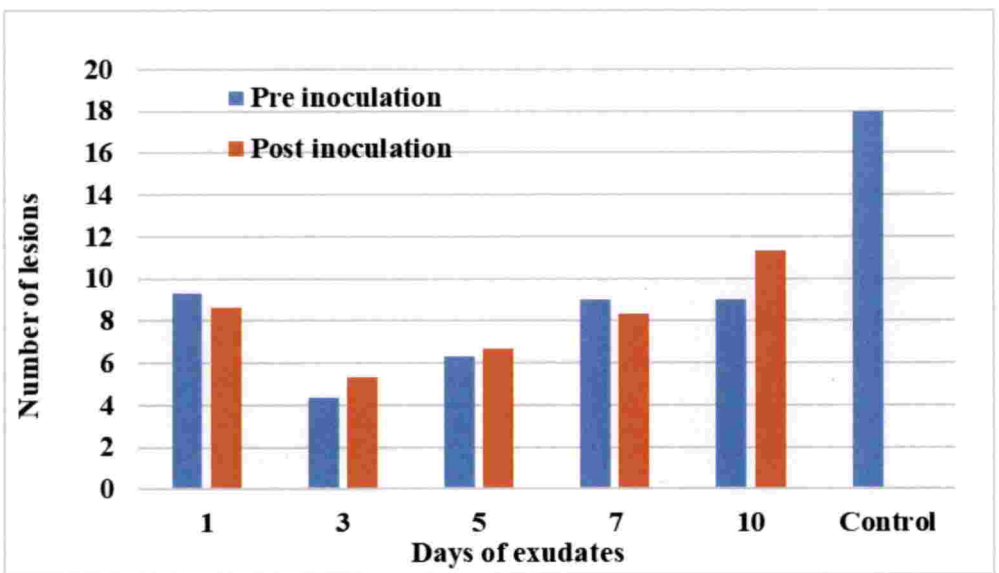


Fig. 12. Inhibition of BlCMV local lesions by *P*i-WDE in *C. amaranticolor*.

roots indirectly mediated resistance against plant virus by producing compounds possessing antiviral property. Anthurium plants colonized with *P. indica* stimulated higher activities of stress related enzymes, jasmonic acids and genes for jasmonic acid mRNAs, which in turn was responsible for resistance to plants against biotic stress (Lin *et al.*, 2019).

5.4.3. Evaluation of *P. indica*-water diffusible exudates on BlCMV in *C. amaranticolor*

The experiment to determine the effect of Pi-WDE on local lesion host, *C. amaranticolor* revealed that all the exudates collected at different intervals inhibited the local lesions with maximum reduction in exudates from three days incubation of mycelium in sterile water (Fig.12). Culture filtrates from *Trichothecium roseum* reduced *Southern bean common mosaic virus* and *Tobacco mosaic virus* with the disease reduction of 99 and 98 per cent respectively (Gupta and Price, 1950). The culture filtrate from *Aspergillus tubingensis* FjBJ11 which contains Malformin A_1 inhibited *Tobacco mosaic virus* in tobacco (Tan *et al.*, 2015). *Centella asiatica* recorded enhanced production of asiaticoside content in *P. indica*-primed plants (Satheesan *et al.*, 2012) and *P. indica* cell wall extract (Jisha *et al.*, 2018) than control. The results suggested that Pi-WDE contain metabolites which functions as that of the fungus in the suppression of BlCMV in the local lesion host plant.

5.5. BIOASSAY IN COWPEA PLANTS AGAINST BlCMV

Potential plant extracts, *P. indica* and Pi-WDE could effectively reduce and inhibit local lesions produced by BlCMV in *C. amaranticolor* were further selected for bioassay in cowpea. Among the plant extracts, *P. niruri* and *B. diffusa* and Pi-WDE from three days incubation of mycelium in sterile water was better in inhibition of BlCMV and therefore used for determining efficacy in cowpea. Treatments were applied as pre and post inoculation. Vulnerability index of blackeye cowpea mosaic disease was calculated and was found that *P. indica*-primed cowpea plants inoculated with the virus were less vulnerable to the virus

infection which might be due to the continous priming of the plants by the fungus whereas the AVPs and Pi-WDE were treated only once. Wang *et al.* (2015) stated that *P. indica* imparts resistance to tomato yellow leaf curl disease in tomato plants by enhanced PAL activities and PR-genes expression and phytohormone signalling mediated resistance. Elsharkway and El-sawy (2015) investigated that all plants possess their own specific mechanism to combat plant viruses. El-sawy *et al.* (2018) identified that the ginger and horsemint extracts and silver nanoparticles acted as elicitors to induce systemic resistance in tomato plants and enhanced peroxidase and polyphenol oxidase activities to suppress symptom expressed by tomato yellow leaf curl disease. Hanaa (2017) reported that the carrageenan (red algae extract) induced resistance in potato by enhanced peroxidase, polyphenol oxidase, superoxide dismutase enzyme activities with increase in total protein, phenol and proline content against *Potato virus Y.* AVPs application may accelerate certain protective substance against plant virus (Prasad *et al.,* 2007). Antiviral activity of *B. diffusa* decreased rapidly and frequent applications were needed for management of plant viral diseases (Kumar and Aswathi, 2007).

The observation on root and shoot biomass of the potential AVPs, *P. indica*, and its WDE treated plants were recorded. *P. indica*-primed virus inoculated plants recorded higher shoot weight compared to *P. niruri* treated and control plants. *P. indica*-primed virus inoculated plants had significantly higher biomass than other treatments imposed plants. *P. indica* directly act on plant hormone signalling pathway to induce IAA, cytokinin and other growth regulators (Johnson *et al.,* 2014). It enhanced root proliferation resulting in better nutrient uptake, increased crop growth and productivity (Lee *et al.,* 2011; Gill *et al.*, 2016; Desia, 2017).

5.6. PHYSICAL AND CHEMICAL PROPERTY OF AVPS

AVPs from *P. niruri* and *B. diffusa* were exposed to moist heat of 121°C for 20 minutes to evaluate its heat stability. The results revealed that there was no significant reduction of local lesion in both moist heat exposed and non-exposed

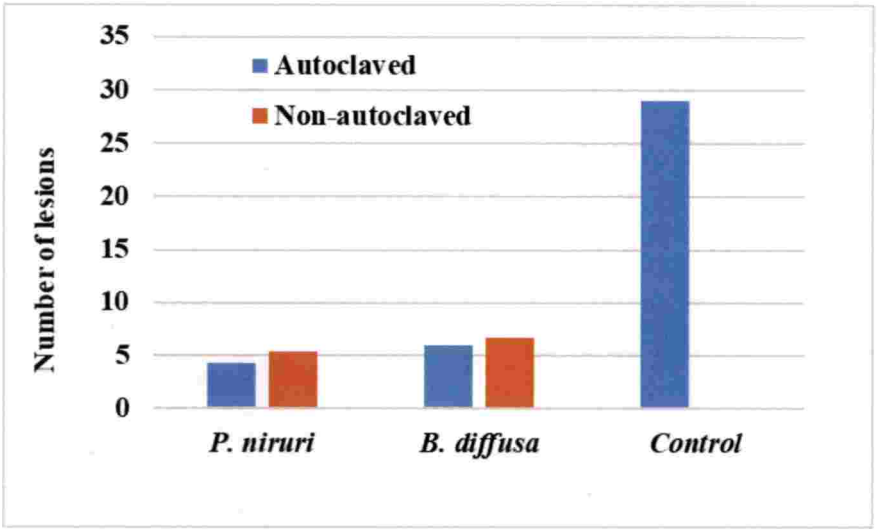


Fig. 13. Inhibition of BlCMV local lesions in *C. amaranticolor* by autoclaved AVPs. of *P. niruri* and *B. diffusa.*

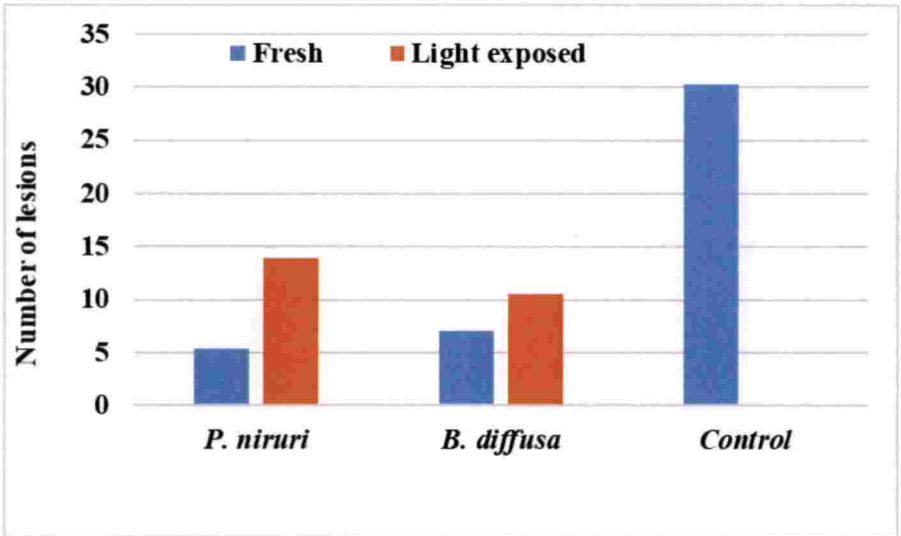


Fig. 14. Inhibition of BlCMV local lesions in *C. amaranticolor* by light exposed AVPs of *P. niruri* and *B. diffusa.*

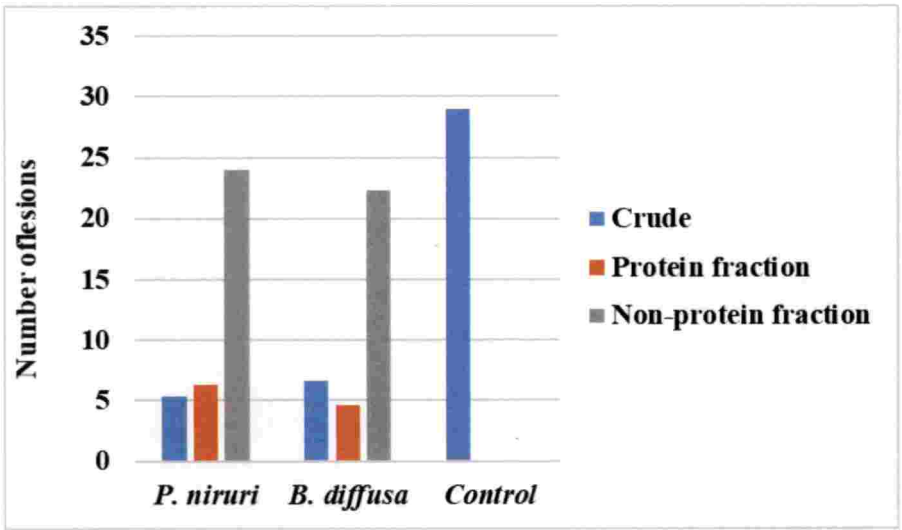


Fig. 15. Inhibition of BlCMV local lesions in *C. amaranticolor* by protein and non-protein fractions of AVPs of *P. niruri* and *B. diffusa*.

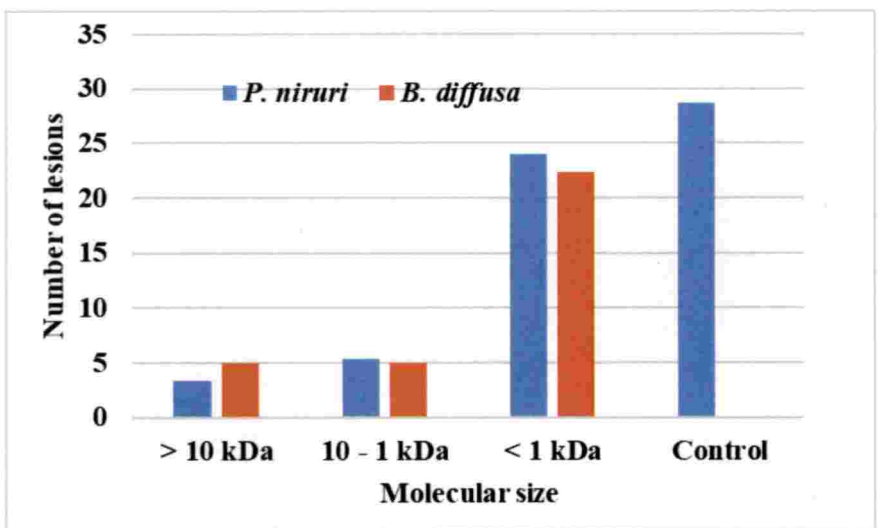


Fig. 16. Inhibition of BlCMV local lesions in *C. amaranticolor* by different molecular sizes of AVPs of *P. niruri* and *B. diffusa*.

AVPs (Fig.13). Leaf extract of *P. niruri* and *B. diffusa* exposed to light for determining its photostability or photosensitive nature. The effectiveness of both the AVPs was reduced than non-exposed AVPs (Fig. 14) as it might have partially degraded due to the exposure to sunlight. It is in line with the findings of Zhou *et al.* (2015), that the photodegradation is the vital process for removal of compounds from the environment. In contradictory to the present findings, Vimalanathan *et al.* (2009) reported that the antiviral activity of plant extracts increased with exposure to photoperiod.

Active principles of *P. niruri* and *B. diffusa* were identified by separating protein and non-protein part from the aqueous extract. The results revealed that the protein part of AVPs reduced the BlCMV infection as that of crude extracts but the activity of non-protein part against the virus is insignificant (Fig.15). This result is in accordance with the previous finding by Bishnoi *et al.* (2017) that the protein part of *B. diffusa* was the antiviral principles inhibiting *Indian citrus ringspot virus*.

Average size of the antiviral proteins in *P. niruri* and *B. diffusa* were determined by passing through molecular sieve of 10 kDa and 1 kDa. The results indicated maximum inhibition of BlCMV local lesion from protein having molecular size of more than 10 kDa and 1 kDa and least inhibition was in less than 1 kDa sieved antiviral proteins (Fig.16). In accordance with present finding, Lohani *et al.* (2007) and Sharma *et al.* (2017) identified that the active glycoprotein of *B. diffusa* correspond to molecular weight of 30 kDa in SDS-PAGE. Roy (2006) identified antiviral principle in *Amaranthus tricolor* against *Sunhemp rosette virus* as glycoprotein with four per cent sugar and the protein part had molecular weight of 27 kDa.

5.7. BIOCHEMICAL CHANGES OF HOST PATHOGEN INTERACTIONS

5.7.1. Total soluble proteins

Quantification of protein content in treated plants revealed that higher content was recorded in virus inoculated *P. indica*-primed plants on 10 DAI and it

was more than that of the healthy and inoculated plants, whereas *P. indica*-primed plants maintained same amount of protein (Fig.17). On 15 DAI, protein content of *P. niruri* and *B. diffusa* treated plants decreased compared to the healthy plants which was in line with the findings of Louis (2003), who reported that *Plumbago rosea* treated plants had reduced protein content. These might be due to conversion of protein into other defense related substance to induce resistance in plants. Low protein content in AVP treated plants might be due to the interference in plant protein synthesis, thus making unfavourable condition for the virus replication (Rajinimala *et al.,* 2009). *P. indica*-colonized plants enhanced the protein content than the uncolonized plants (Krishnaveni *et al.*, 2014). Thuja extracts applied against *Watermelon mosaic virus* in *Citrullus lanatus* reduced the protein content than virus infected plants (Elbeshehy *et al.*, 2015).

5.7.2. Total phenols

Phenols play major role in defense mechanism which determines tolerance or susceptibility of a host-parasitic interaction (Shilpashree, 2006). It was found that phenol content of virus inoculated plants differed significantly from the healthy plants. Phenol content increased steadily with growth of plants and higher accumulation was found in the virus inoculated plants than healthy plants (Fig.18). *P. indica*-primed plants challenged with virus inoculation had gradual increase in phenol content which was higher than healthy and virus inoculated plants. Several research workers reported that the *P. indica* colonized plants of *Aloe vera* (Sharma *et al.*, 2014) and *Stevia rebaudiana* (Kilam *et al.*, 2015) accumulated higher phenol content compared to the uncolonized plants. Veena (2007) observed *P. niruri* and *B. diffusa* treated plants had higher phenol content than healthy and virus inoculated plants. Phenol content of AVPs treated plants increased initially and started to decline which was similar to the observations of Devi *et al.* (2004) and Karthikeyan *et al.* (2009). Louis (2003) explained higher phenol content accumulation produced substances which induce resistance in plants against viral diseases.

5.7.3. Defense related enzymes

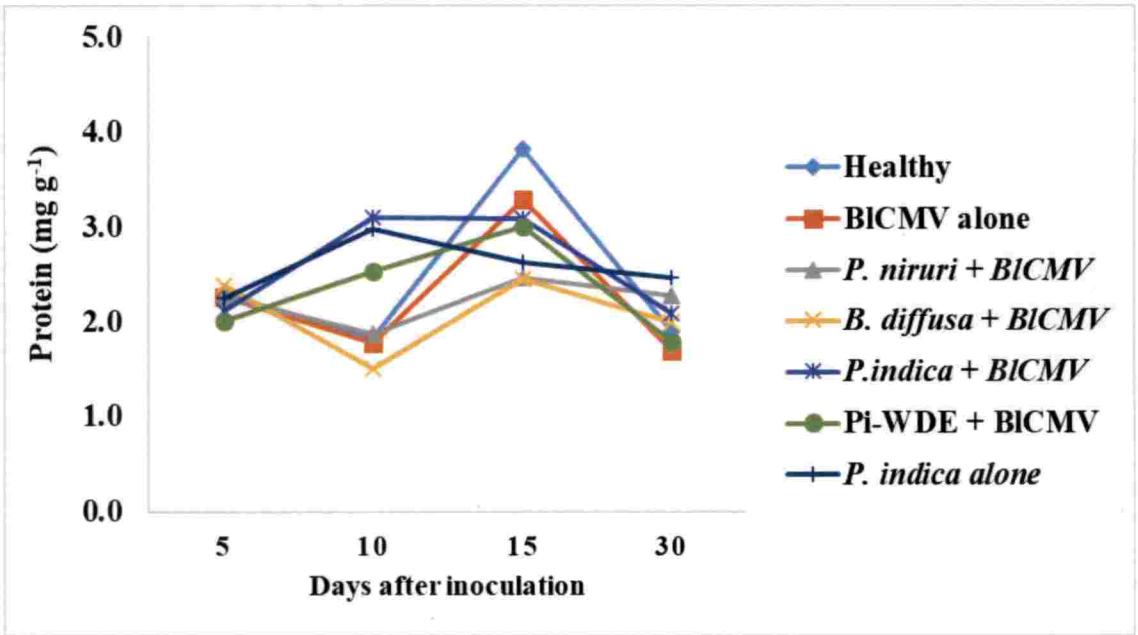


Fig. 17. Changes in total soluble protein content of cowpea leaves treated with AVPs of *P. niruri* and *B. diffusa*, *P. indica*-priming and Pi-WDE against BlCMV.

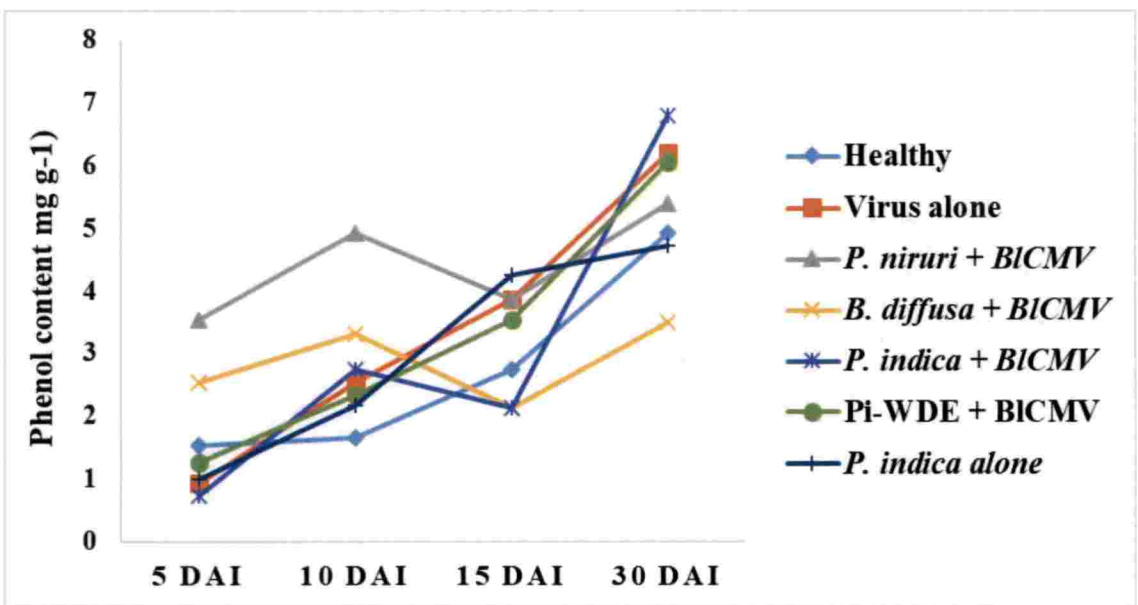


Fig. 18. Changes in total phenol content of cowpea leaves treated with AVPs of *P. niruri* and *B. diffusa*, *P. indica*-priming and Pi-WDE against BlCMV.

During the time of initial virus establishment, various defense related enzymes were expressed to induce resistance in plants. Investigation on enzyme activity clearly indicated that all the treatments induced defense response in plants. PO activity was induced at five DAI and found to be higher in the virus inoculated plants than the absolute control (Fig.19). PO activity increased continuously in *P. niruri* treated plants throughout the study. But in *P. indica* treated plants, it started to decline at 10 DAI but was higher than BlCMV inoculated plants (Fig.19). Alex (2017) observed the enhanced PO activity in *P. niruri* and *P. indica*-primed cowpea plants against BlCMV. Lin *et al* (2019) reported *P. indica* colonisation elicited PO activity in anthurium plants than the un-colonised plants. *P. indica* colonised Chinese cabbage recorded higher PO activity under stress conditions (Sun *et al.*, 2010).

PPO activity of BlCMV inoculated plants increased many times than healthy plants during early symptom expression. On 10 DAI, PPO activity reduced (Fig.20) which might be due to reduced metabolites and destruction of cell organelles (Shilpashree, 2006). *P. indica* treated plant recorded maximum activity of PPO at 10 DAI while, *P. niruri* recorded maximum activity at 15 DAI (Fig. 20). *P. fluorescens* and plant extracts applied against *Urdbean yellow mosaic virus* produced many fold increase in enzyme activity of PO, PPO and PAL in urdbean than healthy plants (Venkatesan *et al.*, 2010).

PAL activity of BlCMV inoculated and healthy plants were insignificant. *P. indica* and its WDE decreased the activity of the enzyme when the plants were challenged with BlCMV (Fig.21). Desia (2017) found that *P. indica* reduced the antioxidant enzyme activity in plants to withstand the effect of the disease. PAL activity was recorded maximum at 5 DAI, which later had shown a decreasing trend with increase in symptom expression in all the treated plants (Fig.21). These findings were supported by Veena (2007) and Alex (2017) in the activity of PAL in BLCMV inoculated cowpea plants. Plantains sprayed with *Pseudomonas* strains CHA0 against BBTV increased the activity of all the three enzymes. Activity of enzymes in bitter gourd plants infected with yellow mosaic diseases

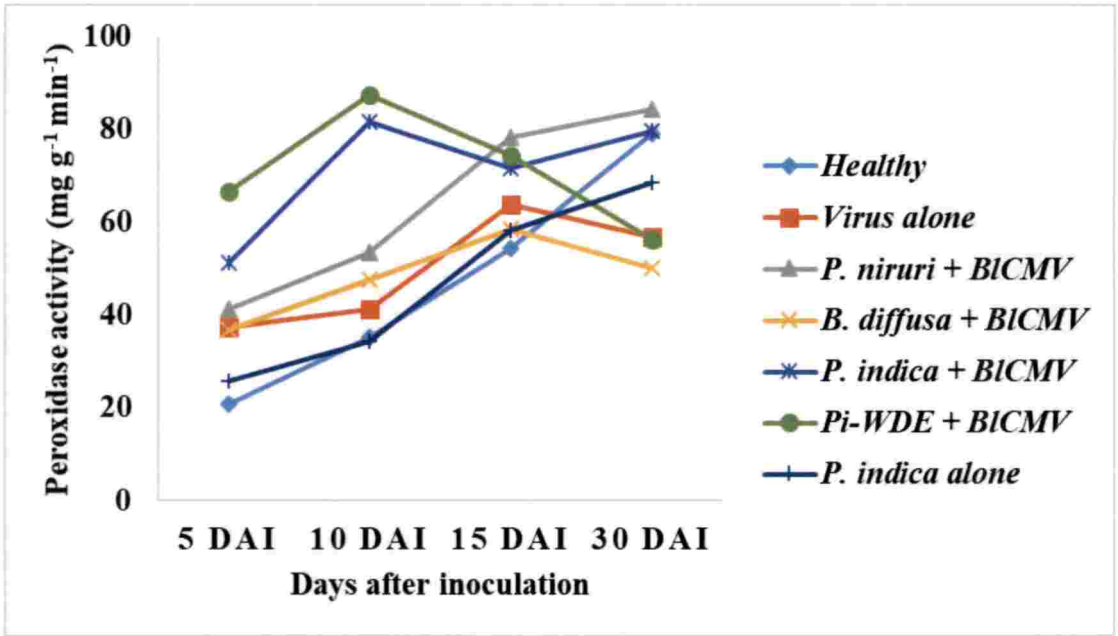


Fig. 19. Changes in peroxidase activity of cowpea leaves treated with AVPs of *P. niruri* and *B. diffusa*, *P. indica*-priming and Pi-WDE against BlCMV.

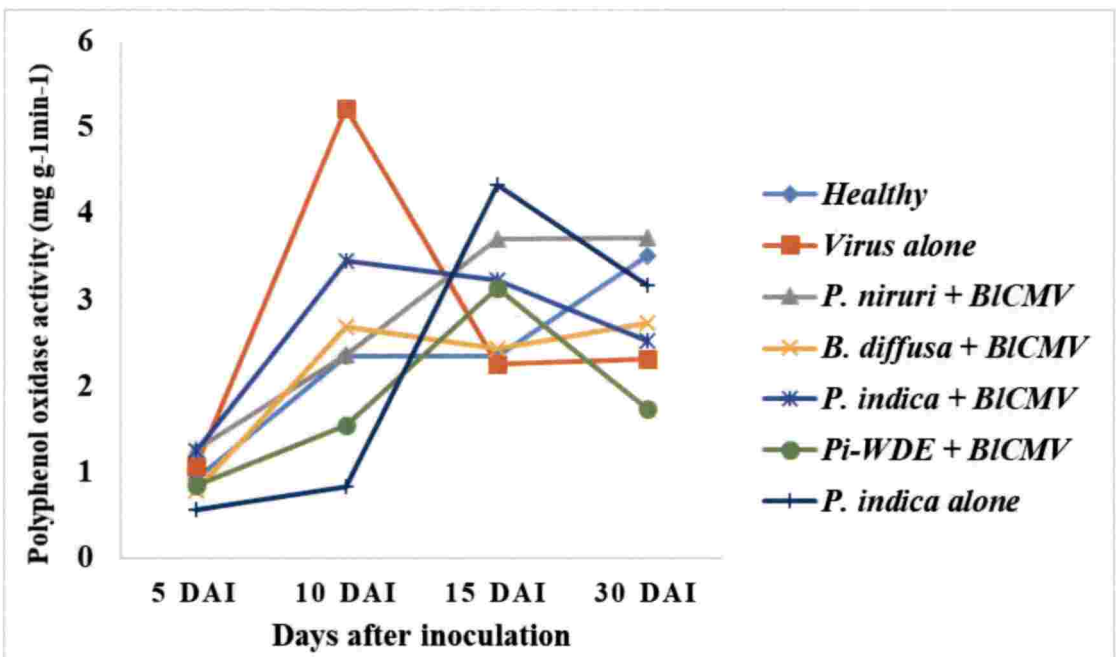


Fig. 20. Changes in polyphenol oxidase activity of cowpea leaves treated with AVPs of *P. niruri* and *B. diffusa*, *P. indica*-priming and Pi-WDE against BlCMV.

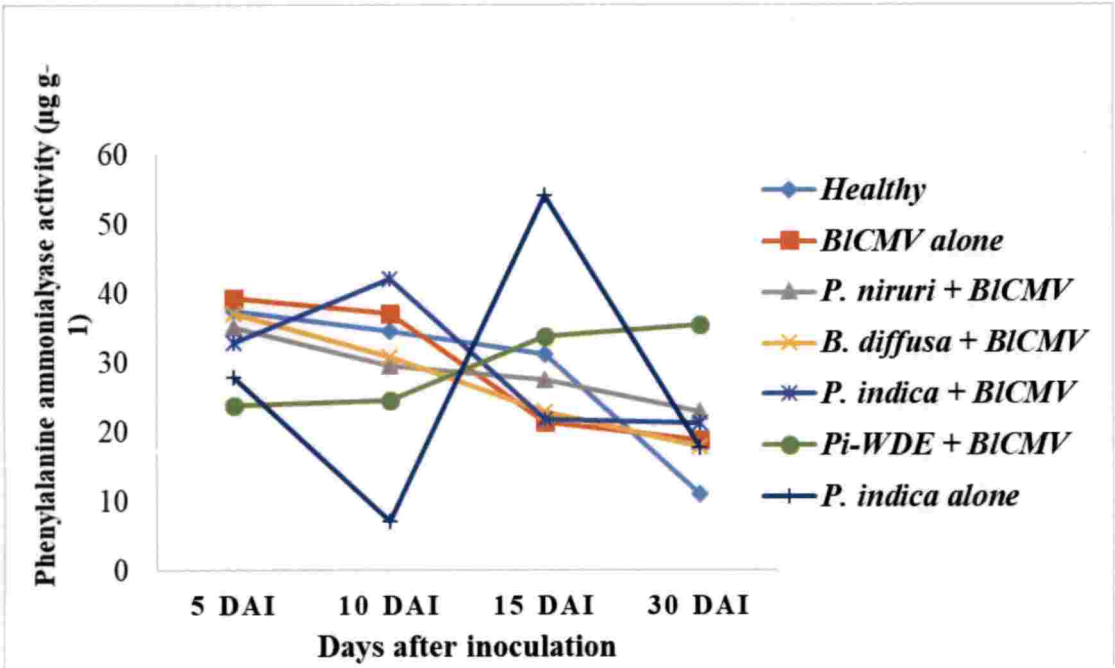


Fig. 21. Changes in phenylalanine ammonialyase activity of cowpea leaves treated with AVPs of *P. niruri* and *B. diffusa*, *P. indica*-priming and Pi-WDE against BlCMV.

was reduced by application of *B. spectabilis*, *Pseudomonas chlororaphis* and *P. fluorescens* (Rajinimala *et al.*, 2009).

PO and PPO enzyme act as catalyst in oxidation of phenols and amines to produce quinones and phenyl propanoid compounds *viz.*, phenolics, phyto alexins and lignin, which imparts resistance to plants (Rajinimala *et al.*, 2009) and PAL play pivotal role in phenyl propanoid pathway (Devi *et al.*, 2004) which in turn enhanced the resistance in plants.

5.7.6. PR- Proteins

Protein profiling of induced PR-proteins in cowpea plants was done with SDS-PAGE in different intervals. PR-proteins analysis revealed that cowpea plants inoculated with BlCMV elicited different proteins and enzymes due to various treatments. Many novel proteins were induced in *P. indica*-primed cowpea plants; 15 bands were observed on 5 DAI. It was in accordance with the findings of Venkatesan *et al.* (2010) that the application of biotic agents (*P. fluorescens*) induced PR-proteins against virus in plants. The induction of PR-protein increased in all treatments from 5 DAI to 15 DAI. *P. niruri* treated plants produced 15 bands while *B. diffusa* treated plants induced 12 bands at 5 DAI. On 30 DAI, PR-proteins induction decreased with reduction in band intensity. It might be due to induction of protein and defense enzymes which could induce resistance in plants and prevented the virus replication in plants (Veena, 2007). Induction of proteins of molecular mass ranging from 30 kDa to 34 kDa in virus inoculated plants was the major and minor protein of BlCMV inoculated plants in SDS-PAGE (Krishnapriya, 2015).

Thus, the present study revealed that *Blackeye cowpea mosaic virus* (BlCMV) in yard long bean could be managed by pre- and post- treatment with 1 per cent AVPs *viz., P. niruri* and *B. diffusa*, and *P. indica*-WDE; and the root colonisation of the beneficial fungal endophyte, *P. indica*. The combined effect of *P. indica* and its WDE with the potential AVPs on management of BlCMV in yard long bean has to be further investigated.

Summary

6. Summary

The research work entitled "Management of *Blackeye cowpea mosaic virus* using natural products from botanicals and the fungal root endophyte *Piriformospora indica*" was conducted during the period of 2017-2019 at the Department of Plant Pathology, College of Agriculture, Vellayani to utilize natural products from potential antiviral plant extracts and the root endophyte *P. indica* for the management of the virus.

Blackeye cowpea mosaic virus was maintained in systemic host, vegetable cowpea (var. Sharika) and the local lesion host, *C. amaranticolor* by sap transmission with phosphate buffer. Symptoms produced by BlCMV in emerging trifoliate leaves include vein clearing, vein banding and blistering, later it developed mosaic, leaf size reduction, leaf distortion, flower malformation, reduced pod length and stunting. It produced yellow chlorotic local lesion progressed to necrotic lesion in inoculated *C. amaranticolor* leaves. Beneficial fungal root endophyte, *P. indica* was maintained in PDA and PDB by frequent sub-culturing as when required. The fungi covered petri plate (9 cm) with PDA in nine days and in broth, 16 days for complete mycelial mat formation.

Serological detection of the virus was done by DAC-ELISA and DIBA. Polyclonal antibodies of CABMV and PVY reacted positively with BlCMV infected leaves confirming the etiology of black-eye cowpea mosaic disease.

Standardization of co-cultivation for *P. indica* with cowpea and *C. amaranticolor* were conducted in both *in vitro* and *in vivo*. The mycelium of *P. indica* was observed to colonize inside cowpea roots within 5 DAC. Chlamydospores were seen on root surface at 7 DAC and chlamydospores inside the roots at 14 DAC in both *in vitro* and *in vivo* methods. *P. indica* took much longer time for the colonization in *C. amaranticolor* roots. Mycelium was observed in roots at one week after co-cultivation (WAC) whereas chlamydospores at two WAC and chlamydospores inside the roots at three WAC in both *in vitro* and *in vivo* study.

Ten plants with reported potential antiviral principles were screened against BlCMV in local lesion host, *C. amaranticolor* at 0.25, 0.5 and 1 per cent with pre- and post- inoculation method. *P. niruri* recorded more than 80 per cent inhibition of BlCMV local lesion in *C. amaranticolor* even at one per cent concentration by pre-inoculation method. *B. diffusa* inhibits 75 per cent of local lesions in *C. amaranticolor* at one per cent concentration in pre-treated plants.

P. indica colonized in roots of *C. amaranticolor* with primed leaves could significantly reduce local lesion caused by BlCMV by 68 per cent over control. Water diffusible exudate from *P. indica* collected at different intervals was evaluated against BlCMV in local lesion host, *C. amaranticolor* with pre- and post- inoculation. Pi-WDE collected after three day mycelium incubation in sterile water was found to inhibit more than 75 per cent of local lesion over control.

Bioassay in cowpea plants with best antiviral principles (*P. niruri* and *B. diffusa*), *P. indica*-priming and Pi-WDE were evaluated against BlCMV. *P. indica*-primed plants were less vulnerable to BlCMV infection followed by *P. niruri* pre-treated plants. BlCMV inoculated in *P. indica*-colonized plants enhanced root (22.7 g plant^{-1}) and shoot (144.8 g plant^{-1}) biomass which was significantly higher than the healthy plants. *P. niruri* pre-treated plants (root – 13.7 g plant^{-1} and shoot 113 g plant^{-1}) were similar in biomass with healthy plants (- control) (root – 18.3 g plant^{-1} and shoot 124.3 g plant^{-1}) while virus inoculated plants (+ control) remarkably reduced root (4.6 g plant^{-1}) and shoot biomass (12.4 g plant^{-1}).

Partial characterization of antiviral principles from best leaf extracts *viz.*, *P. niruri* and *B. diffusa* against BlCMV in *C. amaranticolor* was done. Leaf extracts of both the AVPs were thermostable at 121°C for 20 min and partially photo sensitive (exposed natural light at 9 h). Proteins were the active principles found in the both the AVPs and had a molecular size of more than 1 kDa.

Biochemical changes in host-pathogen interactions of pre-treated AVPs, *P. indica*-colonized and Pi-WDE treated cowpea plants were studied. Protein content

of the virus inoculated plants increased initially up to 15 DAI, thereafter started to decline. Phenol content in healthy and virus inoculated plants increased with age of plants. *P. niruri* and *B. diffusa* treated cowpea plants induced rapid accumulation of phenol up to 10 DAI, later the induction was gradual. All the treated plants with the virus inoculation differs in PO activity, and its enhanced activity was in *P. indica*-primed and *P. niruri* treated plants at 10 DAI, later PO activity gradually declined in *P. indica*-primed while increased in *P. niruri* applied plants. Higher activity of PPO was observed in virus inoculated plants at 10 DAI, and the activity of PPO was significantly different with various treatments. Activity was enhanced during initial period of virus inoculation (upto 15 DAI), then the activity was reduced. Studies on PAL activity in cowpea plants treated with biotic agents and challenged with BlCMV inoculation resulted in the accumulation of PAL within 5 DAI, and there after decreased with age of plants except in Pi-WDE treated cowpea plants which enhanced PAL with age of plants.

Protein profile study (SDS-PAGE) of cowpea plants revealed the induction of many proteins in response to the different treatments. Induction of PR-proteins was more in *P. indica*-primed plants with and without virus inoculation during the initial phase of the tripartite interaction. The intensity of band having molecular weight 29.4 kDa was high in all the treatments except for the virus inoculated plants. An extra band of 24.8 kDa was observed in *P. indica*-colonized virus inoculated plants. *P. niruri* constantly maintained high level of 34 kDa protein in treated plants whereas, in BlCMV treated plants, none of the PR-proteins were induced at different intervals of the experiment.

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Appendices

Appendix -1

Buffer for sap extraction – 0.01M Sodium phosphate buffer (pH 7.0)

Stock solutions:

A: 0.2M solution of monobasic sodium phosphate (2.78g in 100ml)

B: 0.2M solution of dibasic sodium phosphate (5.365g in 100ml)

39 ml of A is mixed with 61.0 ml of B diluted to a total of 200ml.

Appendix – II

Potato Dextrose Agar (PDA) medium

Potato: 200g

Dextrose: 20g

Agar: 20g

Distilled water: 1 litre

Appendix III

Buffers for DAC-ELISA

1. **Phosphate buffer saline (PBS - pH 7.4)**

Sodium chloride	- 8.0g
Potassium dihydrogen phosphate	- 0.2g
Disodium hydrogen phosphate	- 1.1g
Potassium chloride	- 0.2g
Sodium azide	- 0.2g
Water	- 1 L

2. **Wash buffer (PBS-T)**

   Add 250µL of Tween 20 to 500ml of PBS

3. **Coating buffer (pH 9.6)**

Sodium carbonate	- 1.59g
Sodium bicarbonate	- 2.93g
Sodium azide	- 0.2g
Water	- 1 L

4. **Antibody diluents buffer**

   Add 20g Polyvinyl pyrrolidone and 2g ovalbumin to 1L of PBS-T

5. **Enzyme conjugate diluents buffer**

   Same as PBS-TPO

6. **Substrate solution (pH 9.8)**

Diethanolamine	- 97mL
Sodium azide	- 0.2g
Water	- 800 ml

Appendix – IV

Buffers for DIBA

1. **Stock solution buffer (Tris-buffer saline, TBS – pH-7.5)**

   0.02M Tris – 4.84 g

   0.5M NaCl - 58.48g

   Adjust the pH to 7.5 with 1N HCl and make up the volume to 2 litre. This is used as wash solution

2. **Antigen extraction buffer (TBS-SDM)**

   Add 11.25g Diethyl dithiocarbamate (DIECA) to 1 litre TBS

3. **Blocking solution (TBS-SDM)**

   Add 5.0 g spray dried milk (SDM) to 100 ml of TBS

4. **Antibody and enzyme-conjugate diluent/buffer**

   Add antibody of required diluents to TBS-SDM

5. **Substrate buffer (pH 9.5)**

   0.1M Tris - 12.11 g

   0.1M NaCl - 5.85 g

   5mM $MgCl_2.6H_2O$) - 1.01 g

   Adjust the pH to 9.5 with 1N HCl and make up to 1litre.

6. **Substrate solution**

   Solution A

   Nitro blue tetrazolium (NBT) - 75mg

   Dimethyl formamide (DMFA) - 1ml

   Solution B

   Bromo chloro indolyl phosphate (BCIP) - 50 mg

   DMFA - 1 ml

   Store solutions A and B refrigerated in amber colour bottles. Add 44µl of NBT and 35µl of BCIP to 10 ml substrate buffer.

7. **Fixing solution**

   10mM Tris - 1.21 g

   1mM EDTA - 0.29 g

Adjust the pH to 7.5 with 1N HCl and make up to 1litre. All buffers contain 0.02% sodium azide as a preservative.

Appendix V

Murashige and Skoog medium

SI. No.	Chemicals	Stock solutions			For 1 litre
		1 litre	500 ml	250 ml	
Stock 1	NH_4NO_3	82.5 g	41.25 g	20.625 g	20 ml
	KNO_3	95 g	47.5 g	23.75 g	
	KH_2PO_4	8.5 g	4.25 g	2.125 g	
	MgSO4.7H2O	18.5 g	9.25 g	4.625 g	
Stock 2	CaCl. $2H_2O$	22.0 g	11.0 g	5.5 g	20 ml
Stock 3	Na_2.EDTA	3.7 g	1.85 g	0.925 g	10 ml
	$FeSO_4$. $7H_2O$	2.8 g	1.4 g	0.7 g	
Stock 4	$MnSO_4$. $4H_2O$	2.23 g	1.15 g	0.55 g	10 ml
	$ZnSO_4.7H_2O$	0.86 g	0.43 g	0.21 g	
	H_2BO_3	0.62 g	0.31 g	0.15 g	
	KI	0.083 g	0.04 g	0.02 g	
	Na_2MoO_4. $2H_2O$	0.02 g	0.01 g	0.006g	
	$CuSO_4$. $5H_2O$	0.002 g	0.001 g	0.0006 g	
	$CoCl_2$. $6H_2O$	0.0025 g	0.0012 g	0.0006 g	
Stock 5	Glycine	0.2 g	0.1 g	0.05 g	10 ml
	Nicotinic acid	0.5 g	0.025 g	0.012 g	
	Pyridoxim acid. HCl	0.5 g	0.025 g	0.0125 g	

	Thiamine. HCl	0.01 g	0.005 g	0.0025 g	
Inocitol					0.1 g
Sucrose					30 g
Agar					8 g

Above stock solution was pipetted out and 900 ml of distilled water, inocitiol and sucrose were added. pH was adjusted to 5.8 and make upto 1litre.

Appendix VI

Plant Nutrient Medium (PNM)

5 mM KNO3	- 0.5 g
2mM MgSO4. 7H2)	- 0.48 g
2mM Ca(NO3)2	- 0.472 g
Fe. EDTA	- 2.5 ml
Micronutrient mix	- 1 ml
Agar	- 10 g
Distilled water	- 1 litre

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

Micronutrient mix composition

70mM $H3BO_3$

14mM $MnCl_2$. $4H_2O$

0.5mM $CuSO_4$. $5H_2O$

1mM $ZnSO_4$. $7H_2O$

0.2mM Na_2MoO_4. $2H_2O$

10mM NaCl

0.01mM $CoCl_2$. $6H_2O$

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 minutes and make up fnal volume to 450 ml.

Appendix – VII

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 4^0 C.

Appendix – VIII

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. **0.1 M Sodium borate buffer (pH 8.8)**

   Stock solutions

   A: 0.2 M solutions of Boric acid (12.4 g in 1000 ml)

   B: 0.2 M solutions of Borax (19.05 g in 1000 ml)

   50 ml of A is mixed with 30 ml of B, diluted to a total of 200 ml

Appendix IX

ElECTROPHORETIC ANALYSIS OF PROTEIN USING SDS-PAGE

1. **Protein denaturing solution**

   10 M urea - 80 ml

   1 M NaH2PO4.2H2O - 5 ml

   1 M Tris (pH 8) - 1 ml

   5 M sodium chloride - 2 ml

   Make up the volume to 100 ml by adding 12 ml of distilled water

2. **Acrylamide stock (30 %)**

   Acrylamide - 29.2 g

   Bis-Acrylamide - 0.8 g

   Double distilled water - 100 ml

3. **Separating (resolving) gel buffer stock (1.5 MTris-HCl, pH 8.8)**

   Tris base (18.15 g) was dissolved in approximately 50 ml of double distilled water. The pH was adjusted to 8.8 with 6N HCl and volume was made up to 100 ml with diuble distilled water and stored at 4C.

4. **Stacking gel buffer stock (0.5 M Tris-HCl pH 6.8)**

   Tris base (6.0g) was dissolved in approximately 60 ml of distilled water. The pH was adjusted to 6.8 with 6 N HCl and the volume was made up to 100 ml with distilled water and stored at 4C.

5. **Polymerising agents**

   Ammonium per sulphate (APS) – 10% freshly prepared.

   TEMED – Fresh from refrigerator

6. **Electrode buffer (pH 8.3)**

   Tris base - 6.0 g

Glycine	- 28.8 g
SDS	- 2.0 g
Distilled water	- 2 L

7. Sample buffer

Distilled water	- 2.6 ml
0.5 M Tris-HCl (pH 6.8)	- 1.0 ml
2-Mercaptoethanol	- 0.8 ml
Glycerol	- 1.6 ml
SDS 20 per cent (W/V)	- 1.6 ml
0.5 % Bromophenol blue	- 0.4 ml

8. Staining solution

Coomassie brilliant blue R 250	- 0.1 g
Methanol	- 40.0 ml
Glacial acetic acid	- 10.0 ml
Distilled water	- 50.0 ml

9. Destaining solution

As above without Coomasssie brilliant blue R 250

Abstract

Management of *Blackeye cowpea mosaic virus* using natural products from botanicals and the fungal root endophyte *Piriformospora indica*

by

CHANDRAN K.

(2017-11-065)

Abstract of the thesis

Submitted in partial fulfilment of the

requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT PATHOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695522

KERALA, INDIA

2019

ABSTRACT

The study entitled "Management of *Blackeye cowpea mosaic virus* using natural products from botanicals and the fungal root endophyte *Piriformospora indica*" was conducted at Department of Plant Pathology, College of Agriculture, Vellayani during 2017-2019, with the objective to manage *Blackeye cowpea mosaic virus* (BlCMV) using natural products from potential antiviral plant extracts and the fungal root endophyte *P. indica.*

BlCMV was maintained in systemic host, vegetable cowpea (*Vigna unguiculata* var. *sesquipedalis* (L.) Verdcourt) variety Sharika and local lesion host, *Chenopodium amaranticolor* through mechanical / sap inoculation. In vegetable cowpea, initial symptoms like leaf blistering, vein clearing and vein banding were observed at seven days after inoculation (DAI) in newly emerged trifoliate leaves; which was followed by mosaic, vein netting, floral deformation, stunting and yield reduction. The artificially inoculated cowpea plants had a vulnerability index (V.I.) of 83.33 per cent. In *C. amaranticolor,* an average of 30 yellow chlorotic local lesions of size 2-3 mm was recorded at five DAI. Fungal root endophyte, *P. indica* was maintained by periodical sub-culturing in potato dextrose agar (PDA) medium and potato dextrose broth.

BlCMV, a potyvirus was serologically detected using polyclonal antibodies of related potyviruses *viz. Cowpea aphid-borne mosaic virus* (CABMV) and *Potato virus Y* (PVY) (DSMZ, Germany) through Direct antigen coating – Enzyme linked immunosorbent assay (DAC-ELISA) and Dot immunobinding assay (DIBA) as the polyclonal antibody of BlCMV was not commercially available.

Ten plant extracts having potential antiviral principles *viz., Phyllanthus niruri*, *Boerhavia diffusa*, *Datura metel*, *Prosopis juliflora*, *Punica granatum*, *Eucalyptus globulus*, *Cinnamomum cassia*, *Phyllanthus emblica*, *Moringa oleifera* and *Vitex trifolia* were screened at 0.25, 0.50 and 1.0 per cent against pre- and post- inoculation of BlCMV in *C. amaranticolor* in CRD with three replications.

All the selected AVPs were effective in inhibiting / reducing the local lesions formed by BlCMV in *C. amaranticolor* at different concentrations tested. The highest inhibition of the virus was recorded with *P. niruri* (80 %) followed by *B. diffusa, P. emblica* and *M. oleifera* at 1 per cent. Pre-treatment of the plants with AVPs was more effective than post-treatment application.

P. indica root colonization in *C. amaranticolor* which primed the leaves could significantly and substantially reduce / inhibit the local lesions developed by BlCMV. The number of local lesions of the virus was significantly lower (68 % over control) in the *P. indica*-primed plants. Similarly, *P. indica*-water diffusible exudates (*Pi*-WDE) collected at different intervals (1-10 days) also recorded significant reduction of local lesions developed by the pre- and post-inoculation of the virus. *Pi*-WDE extracted after three days of growth recorded maximum reduction of local lesions produced by BlCMV (> 70 % over control). Therefore, *Pi*-WDE has similar functions of *P. indica* and thus, behaves like the fungus.

The best two antiviral principles *viz., P. niruri* and *B. diffusa* at 1 per cent, *P. indica*-priming and *Pi*-WDE significantly reduced the V.I. of the disease in vegetable cowpea var. Sharika in which the virus caused systemic infection. The highest reduction of V.I. of the disease was recorded in *P. indica*-primed leaves (71 % over control) which might be due to the continuous priming of the plants by the fungus whereas the AVPs and Pi-WDE were treated only once. All the experiments were performed in CRD with 3 replications.

Physical and chemical properties of the AVPs of *P. niruri* and *B. diffusa* extracts were studied in *C. amaranticolor*. The results revealed that the AVPs of *P. niruri* and *B. diffusa* were thermostable even at 121^0C and partially photo-sensitive. The AVPs of *P. niruri* and *B. diffusa* were protein(s) and having molecular size of > 1 kDa.

The biochemical analysis of the two antiviral principles *viz., P. niruri* and *B. diffusa*, *P. indica*-priming and *Pi*-WDE mediated tolerance to BlCMV in vegetable cowpea revealed that total soluble proteins, total phenols and activities

of peroxidise, polyphenol oxidase and phenylalanine ammonialyase had no significant role. But the PR-proteins were strongly induced in all treatments except in BlCMV alone. The induction of PR-proteins by the AVPs, *P. indica*-priming and *Pi*-WDE was positively correlated to the enhanced tolerance of cowpea against BlCMV whereas the down-regulation of PR-proteins by BlCMV enhanced its infection in vegetable cowpea.

Thus, the present study revealed that BlCMV could be managed by pre- and post- treatment of vegetable cowpea with 1 per cent AVPs of *P. niruri* and *B. diffusa* or root colonization of *P. indica* or by using *Pi*-WDE which could be further validated under field conditions. The combined effect of *P. indica* and its WDE with the AVPs on management of BlCMV in vegetable cowpea has to be investigated.