# EXPLORATION OF NATURAL PRODUCTS FROM BOTANICALS AND FUNGAL ROOT ENDOPHYTES FOR THE MANAGEMENT OF *Cowpea mosaic virus*

*by*

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# DECLARATION

I, hereby declare that this thesis entitled **"EXPLORATION OF NATURAL PRODUCTS FROM BOTANICALS AND FUNGAL ROOT ENDOPHYTES FOR THE MANAGEMENT OF *Cowpea mosaic virus*"** 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 **"EXPLORATION OF NATURAL PRODUCTS FROM BOTANICALS AND FUNGAL ROOT ENDOPHYTES FOR THE MANAGEMENT OF *Cowpea mosaic virus*"** is a record of research work done independently by Ms. Theresa Alex 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 her.

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## LIST OF ABBREVIATIONS AND SYMBOLS USED

| | |
|---|---|
| % | Per cent |
| L | Litre |
| PDA | Potato dextrose agar |
| mm | Milli meter |
| $^0$C | Degree Celsius |
| CD | Critical difference |
| cm | Centimetre |
| *et al.* | And other co workers |
| G | Gram |
| H | Hours |
| *i. e.* | That is |
| ml | Milli litre |
| Mm | Milli molar |
| kg | Kilo gram |
| min. | Minutes |
| mg | Milli gram |
| Sl. No. | Serial number |
| sp. or spp. | Species (Singular and plural) |
| *Viz.,* | Namely |
| pH | Negative logarithm of hydrogen ions |

| | |
|---|---|
| µm | Micron meter |
| ppm | parts per million |
| wt. | Weight |
| No. | Number |
| Ha | Hectare |
| bp | Base pair |
| kDa | Kilo Dalton |
| kb | Kilo base |
| MW | Molecular weight |
| N | Normal |
| V | Volume |
| M | Molar |
| µl | Micro litre |
| Nm | Nano metre |
| RT-PCR | Reverse transcription-Polymerase chain reaction |
| BCMV | *Bean common mosaic virus* |
| CRD | Completely Randomized Design |
| RNA | Ribonucleic acid |
| BlCMV | *Blackeye cowpea mosaic virus* |
| CABMV | *Cowpea aphid borne mosaic virus* |
| AMF | Arbuscular mycorrhizal fungi |
| CMV | *Cucumber mosaic virus* |
| ELISA | Enzyme linked immunosorbent assay |

| | |
|---|---|
| DIBA | Dot immunobinding assay |
| SDS-PAGE | Sodium dodecyl sulphate-Poly acrylamide gel electrophoresis |
| AVP | Antiviral principle |
| CP | Coat protein |
| PBS-TPO | Phosphate Buffer Saline-Tween Polyvinyl Pyrrolidone Ovalbumin |
| Mab | Monoclonal antibody |
| Pab | Polyclonal antibody |
| DAI | Days after inoculation |
| rpm | Revolutions per minute |
| SDM | Spray dried milk |
| TBS | Tris buffer saline |
| PO | Peroxidase |
| PPO | Polyphenol oxidase |
| PAL | Phenyl alanine ammonialyase |
| NCM | Nitrocellulose membrane |
| NCBI | National Centre for Biotechnology Information |
| BSA | Bovine serum albumin |
| TEMED | Tetramethyl ethylene diamine |

# *Introduction*

# 1. INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) is an important legume crop which is known for its high-quality dietary protein, acceptable palatability and low cost of production (Dhanasekar and Reddy, 2015).

It is a rich source of protein, with 43 and 33 per cent leaf and seed protein content respectively (Nielsen *et al.*, 1993). It is a very important food source in developing countries where animal protein is limited thereby, supplementing the low protein menus due to high cost of animal source of protein (Fawole *et al.*, 2006; Miko and Mohammed, 2007). The crop is well adapted to stress and has excellent nutritional qualities (El- Ameen, 2008). In India, the crop is grown in about 3.9 million ha with an annual production of about 2.21 million tones and average productivity of 683 kg $ha^{-1}$ (Singh *et al.*, 2012).

The major constraints in cowpea production are low yields of traditional varieties, susceptibility to fungal and viral pathogens and insect damage. Among the diseases infecting on cowpea, viral diseases are known to bring an yield loss ranging from 10-100 per cent. Though over 140 viruses have been identified as naturally infecting cowpea (Shoyinka *et al.*, 1997), about 20 viruses possessing RNA genomes are of major occurrence worldwide (Hampton *et al.*, 1997).

Family *Potyviridae* with its six genera, includes the most economically devastating plant viruses. The *potyvirus* group have non enveloped rod shaped flexuous particles 680-900 nm long and 11-13 nm in diameter, 3.4-3.5 nm helix pitch encapsidating a genome of about 9.7 kb with multiple copies of a single protein species of 30 to 47 kDa (Riechmann *et al.*, 1992). *Blackeye cowpea mosaic virus* (BlCMV) and *Cowpea aphid borne mosaic virus* (CABMV) are the two major potyviruses affecting the cowpea production. These viruses can be transmitted by seeds, aphids and by mechanical inoculation.

*Cowpea aphid borne mosaic virus* (CABMV) and *Blackeye cowpea mosaic virus* (BlCMV), members of the *potyvirus* family, are economically significant viral pathogens in Asia (Mali and Kulthe, 1980) and Africa (Taiwo *et al.*, 1982). These viruses have similar physical properties, geographical distribution and symptom production in their host (Taiwo *et al.*, 1982). Both these viruses are seed transmissible as well as transmitted in a non-persistent manner by several aphid species (Atiri *et al.*, 1986) resulting in significant reduction in yield under field condition.

The rhizosphere of both natural and anthropogenic ecosystems is inhabited by a number of mycorrhizal and non-mycorrhizal root-endophytic fungi. These fungal colonization often helps in plant growth and development (bioregulation), plant nutrition (biofertilisation), plant tolerance and resistance to both abiotic and biotic stresses (bioprotection). Therefore, root-endophytic fungi could be used as biological agents to improve plant systems (Franken, 2012).

*Piriformospora indica*, is a novel endophytic root-colonizing fungus isolated from xerophytic plants of Thar Desert, India. Though resembling arbuscular mycorrhizal fungi (AMF) in many aspects, it belongs to the new family Sebacinaceae and new order Sebacinales of Glomeromycota (Weib *et al.*, 2004; Qiang *et al.*, 2011). Similar to AMF, this fungus promotes plant growth, increases the resistance of colonized plants against fungal pathogens and their tolerance to abiotic stress. It also alters the secondary metabolites of many economically important plants and promotes the overall growth and seed production (Harmann, 2011).

Srivastava *et al.* (2011) reported a positive role of *Fusarium pallidoroseum* in plant growth promotion by enhancing the proline content, acid and alkaline phosphomonoesterase activity and peroxidase activity after the inoculation of the fungus into the tomato seedlings.

Management of virus diseases is more difficult than that of diseases caused by other pathogens. Integration of various approaches like the avoidance of sources of infection, control of vectors using chemicals, cultural practices and use of resistant varieties are currently used. Since most of the popular cowpea varieties are susceptible to *Cowpea mosaic virus*, the use of disease resistant varieties is not possible. Furthermore, use of chemicals has its adverse effects on the environment and non-target organisms. Hence an alternative strategy is the activation of natural defense mechanisms in plant for the management of virus disease. *Boerhaavia diffusa* (Thazhuthama), *Phyllanthus niruri* (Keezharnelli) etc., having antiviral principle can be used for activating the defense reactions in plant tissues (Veena, 2007) and several antiviral principles have already reported to be good in managing the virus diseases. The present study is laid out with the following objectives,

1. Study the symptomatology and transmission of cowpea mosaic virus infecting cowpea, along with their biological, immunological and molecular detection which can effectively contribute for the early detection and development of disease management strategies.

2. Use of botanicals and fungal root endophytes for the management of the cowpea mosaic virus.

3. Study the biochemical basis of resistance elicited by the promising botanical and endophyte in the host plant against the virus.

# *Review of Literature*

# 2. REVIEW OF LITERATURE

Cowpea (*Vigna unguiculata* (L.) Walp.) is an important dual purpose legume in the tropics and subtropics. It is native to Central Africa and is a source of dietary protein, fodder crop and also a nitrogen fixer (Adejumo *et al.*, 2001). It is one of the most widely adapted and highly nutritious crop (Popelka *et al.*, 2004).

It represents a rich source of protein, which make it very important as food for human consumption or as animal feed (Ehlers and Hall, 1997). It is an early maturing crop and helps to minimize hunger period that often occurs in farming community prior to harvest (Singh *et al.*, 2002). Vegetable cowpea can be grown throughout the year under Kerala conditions. It is a direct sown crop and can be grown as a floor crop, as an intercrop or a pure crop.

Even though, cowpea is bestowed with tolerance to abiotic factors like drought, they are susceptible to biotic stresses like diseases that impede the expression of its tangible genetic yield potential (Dhanasekar and Reddy, 2015).

## 2.1. MAINTENANCE OF VIRUS AND ENDOPHYTES

### 2.1.1. Cowpea mosaic virus (Cowpea aphid borne mosaic virus (CABMV) / Blackeye cowpea mosaic virus (BlCMV))

Viral diseases are reported to cause significant reduction in yield of cowpea in Asia, Africa and America. Worldwide, more than 20 viruses have been identified as naturally infecting cowpea (Mali and Thottappilly, 1986). Among the viruses that infect cowpea (*V. unguiculata*), *Cowpea aphid borne mosaic virus* (CABMV) and *Blackeye cowpea mosaic virus* (BlCMV) are highlighted for their severity and widespread occurrence.

*Cowpea aphid borne mosaic virus* (CABMV)

Vidano and Conti (1965) were the first to report on CABMV infecting cowpeas (in Italy), and Lovisolo and Conti (1966) described the properties of CABMV. Mali and Kulthae (1980) first reported CABMV in India.

CABMV infects many species in the family Fabaceae (Leguminosae) and most strains also infect members of the Amaranthaceae, Chenopodiaceae,Cucurbitaceae, Labiatae and Solanaceae (Lovisolo and Conti, 1966). Three species in the genus Aphis (*A. craccivora, A. spiraecola* and *A. gossypii*) was found to transmit CABMV efficiently to and from cowpea, bean and soybean host plants (Atiri *et al.*, 1986). A sample of 158 *Vigna unguiculata* plant introductions and germplasm accessions was tested for seed borne potyviruses and a highest percentage of seed transmission were produced with CABMV isolate (Bashir, 1992).

According to Bashir *et al.* (2002) CABMV can cause an yield loss of 13 to 87 per cent under field conditions depending upon crop susceptibility, virus strain and the environmental conditions. Infection resulted in leaf mosaic, vein banding, distortion and stunted growth.

*Blackeye cowpea mosaic virus* (BlCMV)

BlCMV was first reported from the USA in Cowpea cv. California Blackeye No. 5 (Anderson, 1955) and Cowpea cv. California Blackeye No. 5 was found to be susceptible to all BlCMV isolates tested (Kuhn *et al.,* 1965; Lima *et al.,* 1979; Taiwo *et al.,* 1982; Collins *et al.,* 1985). Mali *et al.* (1988) reported that when 60 cultivars from India were screened for the presence of cowpea viruses, BlCMV was identified from 19 based on transmission and serological tests, particle morphology, host range, reactions of cowpea cultivars and properties in crude sap.

*Bean common mosaic virus* strain blackeye cowpea mosaic (BCMV-BlCMV) is an important seed-borne RNA virus infecting cowpea and is transmitted both by seeds and aphids. Infected cowpea seeds can act as primary source of inoculums for disease epidemics (Udayashanker *et al.*, 2010).

Puttaraju *et al.* (2000) reported the widespread occurrence of BlCMV in Karnataka, India. BlCMV was found in 18 fields, out of 21 fields surveyed. Disease incidence in the field varied from 1 to 70 per cent . The yield from the infected fields was estimated to be 50 kg when compared to the expected yield of 2500 kg/ha. The infected plants showed severe green and yellow mosaic, vein banding, blistering and leaf roll. The BlCMV is transmitted by seed-borne transmission with a frequency rate of up to 30% and yield losses as high as 40% under field conditions being recorded (Arakere *et al.*, 2009; Udayashankar *et al.*, 2012).

**2.1.2. Maintenance of Virus**

Cowpea plants were mechanically inoculated with CABMV and the virus were maintained under both field and screen house condition (Ndiaye *et al.*, 1993). Radhika (1999) reported that the *Blackeye cowpea mosaic virus* could be maintained in cowpea and *C. amaranticolor* by sap transmission using 0.01*M* phosphate buffer.

Elbeshey *et al.* (2010) reported that about 90 per cent CABMV transmission and further maintenance was possible by mechanical transmission. Damiri *et al.* (2013) reported the maintenance of CABMV in *V. unguiculata* sub sp. *unguiculata*, *V. unguiculata* sub sp. *sesquipedalis* and *Nicotiana benthamiana* by mechanical inoculation. Krishnapriya (2015) reported that the BlCMV was maintained in a susceptible variety, Vellayani Jyothika on sap transmission using 0.01M phosphate buffer.

Leaves with CABMV symptoms from grown-out cowpea plants were maintained in plant species like *Chenopodium quinoa*, *N. benthamiana, N. tabacum,*

*N.Clevelandii, N. glutinosa, V. unguiculata, Pisum sativum, Glycine max, Solanum lycopersicum* and *Phaseolus vulgaris* by mechanical inoculation (Salem *et al.*, 2010).

BlCMV was maintained in cowpea seedlings by mechanical inoculation with BlCMV infected trifoliate leaves of the highly susceptible "Knuckle Purpose Hull" (Ouattara and Chambliss, 1991). BlCMV was propagated and maintained in yard long bean by mechanical transmission until further use (Huguenot *et al.*, 1993). The stock culture of the BlCMV isolate were maintained by frequent mechanical inoculation on healthy susceptible cowpea plants in an insect-free greenhouse (Bashir *et al.*, 2002) .

Vanilla plant collected from Calicut, Kerala that reacted positively to polyclonal antiserum of BCMV was mechanically inoculated on to *C. amaranticolor* by extracting sap in cold 0.1M phosphate buffer (pH 7.2) containing 0.1% (v/v)2-mercaptoethanol. The extracted sap was inoculated on the leaves of healthy test plants dusted with celite and then washed off with tap water after 2-3 min (Bhadramurthy and Bhat, 2009).

**2.1.3. Symptomatology**

Natural infection with BlCMV / CABMV causes various mosaics, mottling, interveinal chlorosis, green vein banding, leaf distortion, blistering and plant stunting (Bock and Conti, 1974). Bashir (1992) reported mottling, mosaic, vein banding, chlorosis, leaf distortion, necrotic spots, stunting and plant death as symptoms associated with CABMV and BlCMV.

Vein banding, interveinal chlorosis, distortion, blistering and stunting of leaves is observed in the case of CABMV infection (Bashir and Hampton, 1996). Umamaheswaran (1996) described different symptoms of CABMV infections were as prominent mosaic, mottling, chlorosis of leaflets, cupping, arching and inward curling of margins of leaflets, stunting, shortening of internodes, disproportionate branching and deformed pods. CABMV infected (*V. unguiculata*) show variable

amounts of dark green vein banding or interveinal chlorosis, leaf distortion, blistering and stunting (Popelka *et al.*, 2004).

CABMV induces vein banding, mosaic, blisters and stunting in cowpea (*V. unguiculata*) and chlorotic and necrotic local lesions in *C. amranticolor* (Elbeshey, 2010) . Vein clearing, green vein banding, leaf malformation and mosaic symptoms were reported to be the symptoms of CABMV in cowpea (*V. unguiculata*) (Damiri *et al.*, 2013).

BlCMV causes prominent mosaic, mottle, green vein banding and distortion in susceptible genotypes (Pio-Ribeiro *et al.*, 1978). BlCMV produced both localized and systemic symptoms on cowpea. Localized symptoms were large reddish, often ring-like lesions which typically spread along the veins, forming a reddish –net pattern. Systemic symptoms included mottling and green vein banding along with interveinal chlorosis, stunting and leaf distortion (Thottappilly and Rossel, 1985). Sekar and Sulochana (1988) reported that BlCMV infected seedlings showed infection on primary leaves while a few other leaves had yellow patches. Radhika (1999) reported vein clearing as the initial symptom followed by dark green vein banding, severe mosaic, blistering and distortion of leaves and stunting of the plant as the symptoms caused by BlCMV infection. The BlCMV infected seedlings showed mosaic, vein banding, vein clearing, and mottling on the primary leaf followed by mosaic, vein banding, leaf rolling, downward folding of leaves and leaf distortion on the trifoliate leaves (Udayashankar *et al.*, 2009). Krishnapriya (2015) reported that the BlCMV inoculated cowpea plants resulted in a wide range of symtoms including dark green and light green mottling, mosaic and vein banding on leaves, reduction in leaf size and distortion, accompanied with severe stunting of plants with a reduction in number of flowers and pods.

BlCMV was biologically purified through a single local lesion technique on *Chenopodium amaranticolor* by mechanical inoculation (Zettler *et al.*, 1967). Radhika (1999) reported the presence of yellow chlorotic spots on *C. amaranticolor*

inoculated with BlCMV. The leaves of *C. amaranticolor* were inoculated with the BlCMV infected leaf samples from each urd bean samples and the inoculated leaves were observed for local lesions (Udayashankar *et al.*, 2012).

Typical symptoms of seedling infection were downward folding of leaves with conspicuous vein clearing or diffuse chlorotic spots on the first primary leaf, followed by mosaic, vein banding and leaf distortion on the trifoliate leaves. Some of the symptomatic plants were stunted in growth (Hao *et al.*, 2003). *Bean common mosaic virus* (BCMV) induced severe mosaic, vein banding, malformation, leaf curling and stunting on bean plants (Mohammed *et al.*, 2013).

**2.1.4. Maintenance of Fungal Endophytes**

The fungal endophyte *Piriformospora indica,* belonging to Sebacinales in Basidiomycota can be cultivated on complex or minimal substrates (Varma *et al.*, 1999). Vyas *et al.* (2008) reported the maintenance of *P. indica* on Kaefer medium. *P. indica* can be easily cultivated on a variety of synthetic media and helps in improving the plant growth and overall biomass (Oelmuller *et al.*, 2009). Johnson *et al.* (2011) reported the culturing and maintenance of *P. indica* on Kaefer medium (KM). *P. indica* was grown and maintained at $28^0$C in the dark on plates with solid complete medium (Banhara *et al.*, 2015). Varkey (2016) maintained *P. indica* by continuous subculturing on Potato dextrose agar medium. Vyshakhi (2016) reported the isolation and maintainence of fungal endophyte *P. indica,* on potato dextrose agar medium by continuous subculturing.

Praveena and Naseema (2004) conducted the isolation of *Fusarium pallidoroseum* on Potato dextrose agar (PDA) medium and the fungus was maintained on the same media by continuous subculturing. Gokulapalan *et al.* (2006) reported the isolation of *F. pallidoroseum* from symptomatic cowpea samples on Potato dextrose agar medium using standard procedures. Gondim *et al.* (2008) isolated *F. pallidoroseum* from fruit rot infected melon fruits on potato dextrose agar

medium and the fungus was maintained on the same medium by subculturing procedures. The pathogenic *F. pallidoroseum* from infected chilli fruits showing typical fruit rot symptoms was isolated and maintained on PDA medium (Parey *et al.*, 2013).

## 2.2. DETECTION OF *Cowpea mosaic virus* USING SEROLOGICAL METHOD

### 2.2.1. Enzyme Linked Immunosorbent Assay (ELISA)

On the basis of ELISA results, virus isolates of BlCMV and CABMV were identified from germplasm of yard-long bean (*Vigna unguiculata* ssp. *sesquipedalis*) and soyabean plants (*Glycine max*) (Dijkstra *et al.*, 1987). Eighteen isolates of BCMV, five isolates of BlCMV, four isolates of CABMV, and one isolate each of *Adzuki bean mosaic virus* (AzMV) and *Peanut stripe virus* (PStV) were serologically detected and compared using a panel of 13 monoclonal antibodies, in ELISA (Mink and Sibernagel, 1992). Ndiaye *et al.* (1993) used direct antigen coating (DAC) and double antibody sandwich (DAS) ELISA for the detection of seven seed-borne viruses in cowpea seeds.

Bashir and Hampton (1996) used Direct antigen coating-ELISA (DAC-ELISA) and Double antibody sandwich-ELISA (DAS-ELISA) for detection of seed borne viruses in cowpea seeds. Serological test by Plate trapped antigen-ELISA (PTA-ELISA) with group specific antiserum, at a dilution of 1:1500 confirmed the presence of *Potyvirus* infecting cowpea (Sharma *et al.*, 2013).

A biotin/streptavidin ELISA technique was found to be more sensitive than a standard ELISA protocol for detecting CABMV infection in cowpea seeds. The method could detect one CABMV infected seed in a group size of 500 seeds (Konate and Neya, 1996). Ladipo *et al.* (2004) identified a strain of CABMV designated as CABMV-Cr using the serological technique, ELISA with BlCMV and CABMV

antibodies. Taiwo *et al.* (2007) reported that the age of plant at the time of infection and the type of cultivar had significant effect on the titre of CABMV in the infected plants, with highest titre (0.11 to 0.46) detected by ELISA.

Amayo *et al.* (2013) used DAS-ELISA to analyse the seed and leaf samples from CABMV symptomatic and asymptomatic plants. Samples collected from symptomatic cowpea plants grown in the field gave positive reactions with CABMV antibodies but not with BlCMV antibodies in DAS-ELISA, thus confirming that the virus infecting cowpea in kingdom of Saudi Arabia was CABMV (Damiri *et al.*, 2013).

Anderson *et al.* (1996) used ELISA for the evaluation of cowpea lines for resistance against BlCMV. Radhika (1999) used monoclonal antibodies specific to BlCMV (10G5 and 16G5) and CABMV (5H5, 7D9 and 6C10) to detect the mosaic causing virus in cowpea. Three months after inoculation, the symptomless plants were tested for BlCMV by direct antigen coating ELISA (DAC-ELISA) (Bashir *et al.*, 2002). Polyclonal antibodies (PAbs) H2 and monoclonal antibodies (MAbs) 15E6 and 16G5 against BCMV-BlCMV were used for the detection of the virus (Hao *et al.*, 2003).

Representative leaf samples were subjected to DAC-ELISA using antibody raised against *Bean common mosaic virus* strain *Blackeye cowpea mosaic virus* (BCMV-BlCMV ) (Puttaraju *et al.*, 2004). Among the 136 seedlots of cowpea subjected to the growing-on test, seedlings in 43 cowpea seed lots gave positive reactions in ELISA to BCMV-BlCMV (Udayashankar *et al.*, 2009). Krishnapriya (2015) reported a positive reaction of polyclonal and monoclonal antibodies specific to BlCMV confirming the virus causing mosaic disease of cowpea in Kerala as BlCMV.

### 2.2.2. **Dot Immunobinding Assay (DIBA)**

Bhat *et al.* (1999) reported DIBA as the most sensitive detection method for the detection of potyviruses in cowpea. DIBA has been used for CABMV identification in cowpea plants (Sidaros *et al.*, 2006) and it could detect the virus in sample extracts diluted up to 1:1000 (El-Kewey *et al.*, 2007).

DIBA has been used for *Bean common mosaic virus* identification in French bean (Promil *et al.*, 2011). Deepthi and Chalam (2011) reported the virus infecting cowpea as BlCMV, a potyvirus using specific antisera to BlCMV by DIBA. In addition to visual observation, infection of BCMV-BlCMV on test plants was confirmed using Dot immunobinding assay (DIBA) (Melinda, 2013).

According to Huguenot *et al.* (1993) in DIBA, serological identification of *Blackeye cowpea mosaic virus* and *Cowpea aphid borne mosaic virus* are not much easier due to the cross reaction of polyclonal antibodies. Hao *et al.*, 2003 reported a positive reaction to the polyclonal antibodies of both BlCMV (H2) and CABMV (H4) during the serological detection of mosaic causing virus in cowpea. Leaf samples collected from naturally infected cowpea and from those infected cowpea and from those infected via seeds gave positive dot blot hybridization responses while healthy controls were negative (Damiri *et al.*, 2013). Krishnapriya (2015) reported a positive reaction to polyclonal antiserum against BlCMV, thus identifying the virus causing mosaic in cowpea as BlCMV.

## 2.3. DEVELOPMENT OF MOLECULAR PROBE(S) FOR THE DETECTION AND DIAGNOSIS OF *Cowpea mosaic virus*

RT-PCR was performed using specific primer pair, reverse primer and forward primer designed from the coat protein (CP) gene and 3'-untranslated region (Gillaspie *et al.*, 2001). Chen *et al.* (2001) reported that a universal primer deigned

from the consensus sequences that code for the conserved sequence GNNSGQP in the Nib region of members of the family *Potyviridae*, was used to amplify by RT-PCR, the 3'-terminal genome regions from the infected plant samples representing 21 different viruses in the family *Potyviridae.*

Gillaspie *et al.* (2001) reported that RT-PCR detected more virus in seeds infected with CABMV in infected seedlings normally arising in germination tests and was found to be more sensitive than ELISA. EL-kewey *et al.* (2007) used RT-PCR to get a 345 bp size product of coat protein gene of CABMV. Using RT-PCR with virus genus and family specific degenerate primers, CABMV, *Cucumber mosaic virus* (CMV) and *Southern bean mosaic virus* (SBMV) were identified (Salem *et al.*, 2010). Damiri *et al.* (2013) performed RT-PCR for the detection of CABMV isolates in Saudi Arabia using CP gene specific primer which resulted in amplification of 221bp.

Flores *et al.* (2003) designed a set of primers directed to the CP gene to detect and characterize *Bean common mosaic virus* (BCMV) infecting beans in Mexico through RT-PCR. Melgarejo *et al.* (2007) developed a set of degenerate primers for simultaneous detection of BCMV and BCMNV. Udayashankar *et al.* (2009) conducted RT-PCR using both CABMV and BCMV-BlCMV specific primers for the detection of mosaic causing virus in cowpea and an amplification of ~700bp for BCMV-BlCMV indicated the presence of *Blackeye cowpea mosaic virus* in the sample.

Bhadramurthy and Bhat (2009) performed RT-PCR for the detection of BCMV-BlCMV infecting vanilla in Kerala using CP gene specific primer which resulted in amplification of ~850 bp. BlCMV coat protein gene (BlCMV-CP) was amplified at 864 bp from naturally infected cowpea and yard-long bean using CACP1 forward primer and CACP2 reverse primer (Koohapitagtam and Nualsri, 2013). Krishnapriya (2015) performed RT-PCR for the detection of BlCMV and reported

that the CP gene specific primer used for the detection of BlCMV, resulted in an amplicon size of 110 bp.

## 2.4. STANDARDIZATION OF CO-CULTIVATION METHOD FOR FUNGAL ROOT ENDOPHYTES

Endophytes are those microorganisms that reside within growing plant tissues without doing substantive harm or gaining benefit other than residency. Microbial endophytes actively colonize above ground tissues and establish long-term associations, without doing substantive harm to the host. They include bacteria and fungi that can be isolated from surface-disinfected plant tissues or extracted from inside the plant which does not visibly harm the plant. Colonization by endophytes, both fungi and bacteria are reported to enhance the disease resistance of many crop plants (Hallmann *et al.*, 1997). Endophytes are considered to be important candidates for developing into biocontrol agents against plant diseases as they are highly adapted to the host plant system and thus effectively deter the attack of the invading pathogen (Johri, 2006).

*P. indica*, a plant-root colonizing basidiomycetes fungus, has been discovered in the Indian Thar desert and was shown to provide strong growth-promoting activity during its symbiosis with a broad spectrum of plants (Verma *et al.*, 1998). *P.indica* is a wide host root colonizing endophytic fungus which allows the plant to grow under extreme physical and nutrient stress condition .

This fungus functions as a plant growth promoter and biofertilizer in nutrient deficient soils, bio-reactor against biotic and abiotic stress including root and leaf fungal pathogen and insect invaders, bio-regulator for plant growth development such as early flowering, enhanced seed production etc. (Sahay and Varma, 1999). *P. indica*, is a fungus which resembles arbuscular mycorrhizal fungi (AMF) in many

aspects, however, belongs to the new family Sebacinaceae and new order Sebacinales of Glomeromycota (Qiang *et al.*, 2011). Endophytic root colonizing fungus, *P. indica* can be cultivated in vitro unlike the root colonizing AM fungi. *P. indica* colonizes the cortex of plant roots and develops hyphal coils and pear shaped chlamydospores (Varma *et al.*, 2012).

Kumar *et al.* (2009) reported that the fungal endophyte, AMF which are well known for providing a range of benefits to their host range are associated to almost all terrestrial plants except to the plants belonging to some families like *Amaranthaceae, Chenopodiaceae, Cyperaceae, Junaceae* and *Proteaceae.*

Vyas *et al.* (2008) reported that co-cultivation of *P. indica* with *Feronia limonia* roots after 30 days of inoculation resulted in 85 per cent root colonization of *P. indica* within *Feronia limonia* roots . Krishnaveni *et al.* (2015) reported a 55 per cent root colonization in maize roots co-cultivated with *P. indica.* Vyshakhi (2016) reported root colonization of *P. indica* in chilli, tomato and brinjal as 42.03, 81.81 and 42.03 per cent respectively.

Druedge *et al.* (2007) reported that the *P. indica* promotes adventitious root formation in cuttings of vegetatively propagated plants like Pelargonium, Poinsettia and Petunia. Baltruschat *et al.* (2008) reported that in barley total plant fresh weight, leaf length and root fresh weight were significantly elevated after three weeks of inoculation. Besides increasing the growth of plants, the fungus also act against various plant pathogens (Fakhro *et al.*, 2009). Many plants respond positively to inoculation with this fungus and hence, the fungus has multiple biotechnological applications (Oelmuller *et al.*, 2009). Inoculation with the fungus also enhances secondary metabolic production in medicinal plants (Satheesan *et al.*, 2012).

In micropropogated *Artemisia annua, Bacopa monnieri* and tobacco plants, an increase in fresh and dry biomass, plant height and leaf area were observed due to *P. indica* root colonization (Verma *et al.*, 1998). Rai *et al.* (2001) reported a similar

increase in the growth of *Spilanthes calva* and *Withania somnifera* on inoculation with *P. indica*. In micropropagated plants of *Feronia limonia,* a positive influence was observed on vegetative growth and development due to *P. indica* root colonization (Vyas *et al.*, 2008). Hayes *et al.* (2014) reported that *P. indica* inoculated *Medicago truncatula* had a significantly greater number number of roots (102%), total root length (88%), dry weight (25%), heavier shoots (30%), increased leaf area (98%), increased root number (51%) and increased root length (48%) compared to the uninoculated plants and suggested that these physiological and morphological changes may mitigate the limiting factors of crop productivity like biotic and abiotic stresses.

Sharma *et al.* (2014) reported an overall increase in plant biomass in terms of higher shoot and root length, number of shoots and roots in *P. indica* colonized aloe vera plants. Krishnaveni *et al.* (2015) reported an increase in the growth parameters like fresh weight, dry weight, shoot height, root height and leaf length of maize plants colonized with *P. indica* compared to non colonized maize plants. Banhara *et al.* (2015) reported a growth promoting effect of *P. indica* in *Lotus japonicas* and *Arabidopsis thaliana*.

Eighteen day old Arabidopsis seedlings when co-cultivated for nine days resulted in a threefold difference in the fresh weight and a more than twofold difference in the chlorophyll content (Sherameti *et al.*, 2008). Kumar *et al.* (2009) reported 20-30 % colonization at 10 days of *P.indica* co-cultivation in maize plants which later increased to 70 % after 20 days of co-cultivation. Lee *et al.* (2011) reported a bushy root hair pheno-type in *P. indica* colonized chineese cabbage for improved acquisition of water and minerals. Nine day old Arabidopsis seedlings when transferred to KM media containing *P. indica* fungal lawn showed a two fold increase in shoot and root fresh weight after seven days of co-cultivation (Vahabi *et al.*, 2013).

*P. indica* colonized plants results in an increase in fresh and dry weights due to enhanced phosphorous uptake (Sudha *et al.*, 1998). In *P. indica* treated plants, the intensified root proliferation may be due to the synthesis of yet unidentified extra cellular phytohormones (Varma *et al.*, 2001). Rai *et al.* (2001) suggested that the differences in the growth observed between *P. indica* treated and control plants might be due to the greater absorption of water and nutrients due to extensive colonization by *P. indica*. Kumar *et al.* (2009) reported an increase in biomass production of *P. indica* colonized plants compared to uncolonized control plants and this increase in biomass was suggested as the mycorrhiza – like growth promoting activity of *P. indica*.

Sunitha (1997) reported both the spore suspension and wettable powder formulation of *Fusarium pallidoroseum* as effective against Pea aphid. The study also revealed the non pathogenicity of *F. pallidoroseum* towards vegetables *viz.*, amaranthus, bhindi, bittergourd, brinjal, chillies, snakegourd, tomato, and medicinal plants, *viz.*, adathoda, notchi and ocimum under field conditions. Praveena and Naseema (2004) reported the use of *Fusarium pallidoroseum* as a biocontrol agent of water hyacinth resulting in the drying up of plants. The study also revealed *Fusarium pallidoroseum* as a pathogen of amaranthus, tomato, papaya, banana, colocasia and cashew. Gokulapalan *et al.* (2006) reported the *Fusarium* spp. causing wilt in cowpea as *Fusarium pallidoroseum* causing yellowing, withering, drooping of leaves, blackening and drying of veins with an occasional symptom of size reduction and sterility of flowers causing severe yield reduction. *F. pallidoroseum* causes fruit rot disease in melon fruits (Gondim *et al.*, 2008). The pathogenic *F. pallidoroseum* were isolated from blighted maize plants (Orole and Adejumo, 2009). The pathogenic *F. pallidoroseum* was isolated from infected chilli fruits showing typical fruit rot symptoms (Parey *et al.*, 2013).

Srivastava *et al.* (2011) reported that *F. pallidoroseum* a saprophytic fungus, which on inoculation to tomato seeds showed a significant plant growth promotion by

enhancing the proline content, acid and alkaline phosphomonoesterase activity and peroxidase activity. Also an enhancement in shoot dry weight and shoot length of wheat, maize, marigold, okra, mungbean and brinjal over control was also reported.

## 2.5. MANAGEMENT OF BICMV USING BOTANICALS AND FUNGAL ROOT ENDOPHYTES

An extract from dried roots of mature plants of *Boerhaavia diffusa* or a glycoprotein isolated therefrom, when sprayed on leaves of test plants before inoculation, prevented infection by *Tobacco mosaic virus* (TMV) in tobacco, *Cucumber mosaic* and *Tobacco mosaic viruses* in tomato, *Cucumber green mottle virus* in melon, *Sunhemp rosette virus* in *Crotolaria juncea* and *Gomphrena mosaic virus* in *Gomphrena globosa* (Aswathi *et al.*, 1984). Aswathi and Rizvi (1999) reported that the purified glycoprotein from *B. diffusa* reduced infection and multiplication of tomato yellow leaf curl virus. The purified glycoprotein from *B. diffusa* reduced infection and multiplication of *Papaya ring spot virus* (Aswathi, 2000).

The purified glycoprotein from *B. diffusa* reduced infection and multiplication of *Cucumber green mottle mosaic virus* (Aswathi *et al.*, 2003). Singh *et al.* (2004) reported that the destructive yellow mosaic disease of mungbean was prevented under natural field conditions by spraying clarified aqueous root extract of *B. diffusa*. Goyal *et al.* (2010) reported that root of *B. diffusa* contains basal proteins which show high virus inhibitory activity against plant viruses. Root extract of this plant induce strong systemic resistance in susceptible host plant. Rajpoot and Mishra (2011) reported that roots of *B. diffusa* contains basal proteins which show high virus inhibitory activity against plant viruses. Root extract of this plant induce strong systemic resistance in susceptible host plant.

Pre-inoculation of medicinal plant extracts such as *Phyllanthus fraternus, Plumbago roseas* and *Thespesia populnea* was better than the post inoculation which shows inhibition of TMV infection (Louis and Balakrishnan, 1996). Gujetti and Mamidala (2015) conducted a study to evaluate the cytotoxic effects and anti-HIV activity of *P. niruri* whole plant extracts and found that chloroform and hexane extracts of *P. niruri* showed highest antiviral activity. *P. niruri* and *B. diffusa* were shown to inhibit CABMV in pre, post and simultaneous inoculation even at 1% concentration (Veena, 2007).

Mandal and Singh (2001) reported that tender *Psidium guajava* leaf extract had inhibitory effect on the sap transmission of *Chilli mosaic virus* and a greater inhibitory action was observed when it was mixed with the virus inoculum. The acetone-precipitated fractions of *P. guajava* were found effective in reducing the infection by TMV (Deepthi *et al.*, 2007). The aqueous leaf extracts of *P. guajava*, *Phyllanthus niruri* and *Thuja occidentalis* were found to be effective in reducing the infection by *Tomato mosaic virus* (ToMV) (Deepthi *et al.*, 2007). Prasad *et al.* (2007) found that leaf extracts of *B. diffusa* and *P. guajava* contain antiviral principles which inhibited infection by *Blackeye cowpea mosaic virus* in cowpea. Chollom *et al.* (2012) demonstrated that *P. guajava* leaf extract has nutritional value as well as great antiviral activity.

Ali *et al.* (1996) observed antiviral activity for *Calotropis gigantea* and *Euphorbia hirta* while screening ethanolic extracts of 61 medicinal plants for antiviral and cytotoxic activities. When leaf extract of *E. hirta* was mixed with crude preparations of *Tobacco mosaic virus, Sunnhemp rosette virus, Gomphrena mosaic virus* or *Tobacco ring spot virus* and applied to leaves of several hypersensitive hosts, the extract inhibited infection, evidently by increasing host resistance (Verma and Aswathi, 1979). Gyuris *et al.* (2009) reported that the extracts of *E. hirta* have antiretroviral activities on HIV-1 and HIV-2.

Denyer *et al.* (1994) reported that the sesquiterpenes of *Zingiber officinale* displayed antirhinoviral effects. Sydiskis *et al.* (1991) reported that *Aloe vera* contains the antiviral property, like aloe emodin which makes certain viruses not functional. *A. vera* provides a viracudal to *Herpes simplex virus* type 1 and type 2, *Varicella-Zoster virus*, *Pseudorabies virus* and *Influenza virus*.

Some of the evidences showed that in many cases, viral inhibition is due to the development of virus inhibitory substances within the tissues, but some induces systemic resistance (Verma and Prasad, 1984). Pre inoculation application of plant extracts was found to be more effective than post inoculation application in reducing incidence of *Cowpea mosaic virus* (Mallika Devi, 1990). This reduction in vulnerability of cowpea plants may be due to increased activity of phenols, peroxidase, polyphenol oxidase and phenylalanine ammonialyase (Renukadevi *et al.*, 2004). Prasad *et al.* (2007) evaluated the efficacy of certain plant extracts in reducing *Bean common mosaic virus* strain *Blackeye cowpea mosaic virus* (BCMV-BlCMV) in cowpea and found that when plant extracts were mixed with BCMV-BlCMV inoculums, a reduction in disease incidence was observed under greenhouse conditions when compared to control.

Plant extracts stimulate the hosts to produce virus inhibitory agents (VIAs) that spread to surrounding tissues and other plant parts (Verma *et al.*, 1996). Verma and Aswathi (1980) reported the inactivation of viruses *in vitro* using the virus inhibitory agents (VIAs) isolated from leaves of plants treated with phytoproteins. Plant extracts operates induced resistance through the activation of natural defence mechanisms of the host plant and induces systemic resistance to viral multiplication in plants. The active products present in these extracts have no direct effect on viruses; their antiviral activity is mediated by host cells in which they induce the antiviral state. Systemic resistance inducers obtained from plant extracts was also found to be effective against a wide range of viruses (Verma and Baranwal, 2011) .

Waller *et al.* (2005) reported that *P. indica* colonized barley plants showed higher resistance to pathogens with an increased yield. The colonization of maize plants by *P. indica* leads to increased growth, systemic resistance to biotic stress and enhanced antioxidant capacity (kumar *et al.*, 2009). *P. indica* induces enhanced growth with improved resistance against biotic and abiotic stress in barley (Achats *et al.*, 2010). Field trials on *Lagenaria siceraria* and *Tagetes* sp. showed that interaction with *P. indica* suppressed the infestation by plant pathogens including viruses (Shrivastava and Varma, 2014).

## 2.6. BIOCHEMICAL CHANGES OF HOST PATHOGEN INTERACTION

### 2.6.1. Estimation of Protein

An increase in protein level in virus infected plants have also been reported by several workers (Chakraborty *et al.*, 1993; Shivaprasad *et al.*, 2005).

Padma *et al.* (1976) reported that *Cowpea mosaic virus* infected seeds contained a higher percentage of proteins, than healthy seeds. Singh *et al.* (1978) found that *Southern bean mosaic virus* infection resulted in higher total nitrogen, total protein, nitrate and nitrite nitrogen than in healthy leaves of cowpea. Singh and Singh (1981) while investigating the changes in nitrogenous constituents of cowpea pods due to *Cowpea mosaic virus* infection found that there was an increase in total nitrogen, protein and nitrate nitrogen.

Ahmed *et al.* (1992) reported that total protein and soluble proteins were found high in virus free resistant varieties. Thind *et al.* (1996) reported that *Mungbean yellow mosaic virus* (MYMV) infection on mungbean lowered the protein content in leaves. Mali *et al.* (2000) reported that free aminoacid and soluble protein content increased levels of MYMV infection on susceptible varieties of mungbean. Manickam *et al.* (2000) studied the impact of application of a foliar spray of AVPs from *Cocos nucifera, Sorghum vulgare, Sorghum bicolor* and *Croton sparsiflorus*

leaves and inoculation of TSWV on the non-reducing sugar and total soluble protein contents of cowpea plants. It was found that AVP treated cowpea plants showed marginal increase in protein contents compared to significant increase in TSWV inoculated plants. They also studied the effect of foliar spray of AVPs and inoculation of TSWV on the RNA content of *Vigna unguiculata* and *Vigna radiata* plants.

Sindhu (2001) reported that BlCMV infection resulted in increased total soluble protein content in resistant genotype. The leaves of *Bean yellow mosaic virus* (BYMV) infected *Phaseolus vulgaris* showed a reduction in amino acid content of 50 and 77 per cent at 12 and 20 days after infection, respectively (Hemida, 2005). Langhams and Glover (2005) reported, 0.2 to 18.5 per cent increase in seed protein of winter wheat inoculated with *Wheat streak mosaic virus* (WSMV). Taiwo *et al.* (2007) demonstrated that individual infection with CABMV, *Cowpea mottle virus* (CPMoV) and *Southern bean mosaic virus* (SBMV) as well as mixed infection, lowered the protein content of the seeds of all the cowpea cultivars and lines by 24.8 to 28.9 per cent. Yellow vein mosaic disease of mesta cause a reduction in phenolic content (Chatterjee and Ghosh, 2008).

Ashfaq *et al.* (2010) studied the effect of viral infection on total soluble proteins and antioxidant enzyme activity in two genotypes *viz.*, Mash 88 susceptible and CM-2002-resistant, at different stages under both the inoculated and uninoculated conditions and resulted in significant increase in total soluble protein contents of the leaves in both genotypes. Sinha and Srevastava (2010) indicated an increase in total protein content (1.64 to 1.99 mg dry weight) in MYMV infected mungbean plants than their healthy counterparts (1.11 to 1.59 mg per 100 mg dry weight). Srivastava and Singh (2010) reported a remarkable increase in protein content due to *Urdbean leaf crinkle virus* infection in both the cultivars T-9 and IPU 94-1.

*Cassava mosaic virus* (CMV) infection in cassava resulted in the reduction of total soluble protein content in cassava plants (Philip, 2010). Mohammed (2011) reported that *Beet mosaic virus* (BtMV) inoculated Beet plants (*Beta vulgaris* L.) recorded 7.93, 8.53 and 19.27 per cent increase in protein content over healthy plants at first, second and third week respectively after inoculation. Analysis of pathogen related proteins revealed that the content of such proteins was greater in the diseased plants with both *Bean yellow mosaic virus* (BYMV) and *Broad bean stain virus* (BBSV) (Mahfouze *et al.*, 2012). Krishnapriya (2015) reported a significant increase in protein content in BlCMV inoculated cowpea plants without any significant difference in protein content of healthy cowpea plants.

Vyas *et al.* (2008) reported that *in vitro* raised plantlets of *Feronia limonia* (L.) Swingle when colonized with *P. indica* revealed an increase in total protein content compared to the uncolonized control plants.

### 2.6.2. Estimation of Total Sugars

An increase in total sugar content was reported by many workers in virus infected plants (Sohal and Bajaj, 1993; Prasad *et al.*, 1995). Singh and Singh (1980) reported a decrease of total sugar and starch in leaf tissues of sunhemp infected with *Bean mosaic virus*. Singh and Singh (1984) observed that the virus infection decreased total sugar and starch in cowpea cultivars infected with *Southern bean mosaic virus* and *Cowpea mosaic virus*. Bensal *et al.* (1990) found that there was significant increase in amount of total sugars, amino acid and phenol in virus infected plants sprayed with sorghum leaf extract.

Thind *et al.* (1996) reported that the amount of total sugars and starch decreased in black gram infected with *Yellow mosaic virus* when compared to healthy plants. Bhagat and Yadav (1997) reported that healthy leaves of susceptible and

highly cultivars showed higher content of total sugar than resistant one in the case of *Bhindi yellow vein mosaic virus* infected plants. It was also reported that increased sugar content in inoculated leaves of bhindi was due to their accumulation, as a result of the disruption of normal phloem transport. Mali *et al.* (2000) reported that *Mungbean yellow mosaic virus* (MYMV) infection on moth bean resulted in significant reduction of total soluble carbohydrates in susceptible cultivar (GMO 9101) when compared to resistant cultivar (CZM 79). Sindhu (2001) also reported a reduction in the level of carbohydrate content in susceptible varieties inoculated with BlCMV compared to healthy control. Total sugar content was increased in CMV infected tapioca leaves showing an increase of 12.8% (Philip, 2010). Krishnapriya, 2015 reported that BlCMV infection in cowpea plants showed a significant decrease in total sugar content when compared to healthy plants.

Shrivastava and Varma (2014) reported around 16 % increase in sugar content in *P. indica* treated plants. Das *et al.* (2014) revealed an increased sugar content in aerobic rice inoculated with *P. indica* compared to the control ones. Ghabooli (2014) revealed that *P. indica* increases the sugar content in inoculated plants compared to the control plants under salt stress.

### 2.6.3. Estimation of Reducing Sugars

Khatri and Chenulu (1969) reported that reducing sugar content was not appreciably affected by *Cowpea mosaic virus* in resistant and susceptible cowpea cultivars. *Yellow vein mosaic virus* infection reduced the chemical constituents of bhindi like reducing sugar, total sugar etc. (Sarma *et al.*, 1995). Dantre *et al.* (1996) reported that in the case of *Yellow mosaic virus* infecting soybean, reducing sugar, non-reducing sugar and total sugar decreased in infected leaves.

Thind *et al.* (1996) reported that the amount of reducing sugars decreased in black gram infected with *Yellow mosaic virus* when compared to healthy plants. Bhagat and Yadav (1997) reported that healthy leaves of susceptible and highly

cultivars showed higher content of reducing and non-reducing than resistant one in the case of *Bhindi yellow vein mosaic virus* infected plants. Veena (2007) reported higher values of reducing sugar content for AVP treated cowpea plants and assumed that the increased sugar content in treated plants might be due to its accumulation as a result of the disruption of normal phloem transport.

### 2.6.4. Estimation of Phenol

Ramiah (1978) found that there was no difference in phenol content between healthy and inoculated leaves of MS 9804 and CO-1. It was reported that in the variety CO-2 the virus inoculated leaves had higher content of phenol than that of healthy leaves at 40 days after inoculation with *Cowpea aphid-borne mosaic virus*. Sharma *et al.* (1984) studied the effect of virus and fungus infection in muskmelon and showed an increasing trend of the enzyme activity and phenol component as compared to healthy control irrespective of the nature of infection. Rathi *et al.* (1986) assayed total phenol in pigeon pea cultivars resistant and susceptible to *Sterility mosaic virus* and reported that phenolic content increased in both resistant and susceptible varieties. Sastry and Nayadu (1988) recorded higher quantities of phenolic compounds in hypersensitive cowpea leaves infected with *Tobacco ring spot virus*.

Sohal and Bajaj (1993) reported an increase in total phenols in both resistant and susceptible varieties of black gram infected with *Yellow mosaic virus*. Sutha *et al.* (1997) found that both total phenol and *ortho*-dihydroxy phenol increased in *Tomato spotted wilt virus* (TSWV) infected plants. Resistant cultivars had higher content of phenol, OD-phenol and flavanol due to *Cotton leaf curl virus* infection when compared to susceptible varieties (Kaur *et al.*, 1998). Sutha *et al.* (1997) reported that TWSV infection reduced the concentration of total and *ortho*-dihydroxy phenol contents of tomato fruits. Singh and Srivastav (1999) reported

that CABMV virus infection increased the total phenols in diseased leaf compared to healthy ones. Radhika (1999) reported that there was not much change in phenol content in both resistant and susceptible varieties of cowpea infected with *Black eye cowpea mosaic virus*.

Mali *et al.* (2000) reported that *ortho*-dihydroxy phenol was higher in healthy leaves than diseased leaves in case of *Yellow mosaic virus* infected moth bean. Veena (2007) reported a multifold increase in the phenolic content of AVP treated cowpea plants compared to healthy and CABMV inoculated cowpea plants and explained that the increase may be due to increased phenolic accumulation may be due to the excess production of $H_2O_2$ via increased respiration or due to the activation of hexose - monophosphate pathway, acetate pathway and release of bound phenols by hydrolytic enzymes. Yellow vein mosaic disease of mesta cause a reduction in total soluble protein (Chatterjee and Ghosh, 2008).

Following the pathogen infection, phenol synthesizing pathway are accelerated thereby resulting in the increased levels of phenol (Meena *et al.*, 2008). Shilpasree *et al.* (2012) investigated on changes in phenol content between resistant and susceptible genotypes of cowpea, DCS-6 and GC-3 respectively. With increase in age of the plant from 30 to 60 days, the phenol content increased (0.51 to 0.57 mg per g fresh weight) in the susceptible genotype, whereas, there was depletion of total phenols (0.71 to 0.52 mg per g fresh weight) in the resistant genotype.

*Urdbean leaf crinkle virus* infection resulted in significant increment in total soluble phenols in susceptible genotype (Ashfaq *et al.*, 2014). No statistically significant differences were observed in total phenol and flavonoid levels between healthy and CABMV infected passion fruit plants (Tomomitsu *et al.*, 2014). Krishnapriya ( 2015) reported a higher phenol content for BlCMV inoculated control plants compared to healthy cowpea plants in case of both resistant and susceptible variety.

Tanha *et al.* (2014) reported no significant difference in Phenol content of *P. indica* colonized globe artichoke Plants when compared to control. Symbiotic association of *P. indica* with the medicinal plant *Aloe vera* resulted in an increase in phenol content than the uninoculated control plants (Sharma *et al.*, 2014). An increase in total phenol content was observed in *Pennisetum glaucum* seeds treated with *P. indica* than the control plants (Mishra *et al.*, 2014).

**2.6.5. Estimation of Defence Related Enzymes**

Khatri and Chenulu (1970) studied the changes in the peroxidase (PO) enzyme activity in leaves of resistant and susceptible cowpea varieties and observed that the peroxidase activity increased in both resistant and susceptible variety with a higher value for susceptible variety. Mali *et al.*(2000) reported that the activity of catalase, peroxidase and nitrate reductase was found to reduce with increased intensity of disease in the case of yellow mosaic disease of moth bean (*Vigna aconitifolia*). An increase in peroxidase activity was observed in *P. indica* colonized *Feronia limonia* plants compared to the non colonized plants (Vyas *et al.*, 2008).

Sohal and Bajaj (1993) reported an increase in polyphenol oxidase (PPO) activity in resistant variety of black gram infected with *Yellow mosaic virus*. Ashfaq *et al.* (2010) studied the effect of viral infection on total soluble proteins and antioxidant enzyme activity in two genotypes *viz.,* Mash 88 susceptible and CM-2002-resistant, at different stages under both the inoculated and uninoculated conditions and resulted that the activity of PPO increased significantly after 15 and 30 days of inoculation in resistant genotype.

Batra and Kuhn (1975) found that when primary leaves of hypersensitive soybean plants were infected with *Cowpea chlorotic mottle virus*, the enzymes

polyphenol oxidase and peroxidase increased 2-3 times over healthy plants. They also found that the increase was concomitant with the development of acquired resistance. Rathi *et al.* (1986) assayed peroxidase, polyphenol oxidase and isozyme of peroxidase in pigeon pea resistant and susceptible cultivars to *Sterility mosaic virus* and noted that there was not much difference between two varieties with respect to peroxidase and polyphenol oxidase activities. Resistance was characterized by the presence of specific isoperoxidase and proteins.

Wagih and Coutts (1982) reported that *Tobacco necrosis virus* infected cowpea and cucumber showed increase in the amount of extractable peroxidase and polyphenol oxidase activity. Ahmed *et al.* (1992) found that peroxidase and polyphenol oxidase showed no significant difference in virus free susceptible and resistant plants while studying biochemical basis of resistance to *Yellow vein mosaic virus* in okra. Ming *et al.* (1999) observed that, after inoculation with *Soybean mosaic virus* (SMV), peroxidase activity increased a little in the susceptible varieties, but both peroxidase and polyphenol oxidase activities were rapidly decreased in the seed coat of resistant varieties. The resistance thus induced was due to increased activity of catalase, peroxidase and polyphenol oxidase. (Chatterjee and Ghosh, 2008) reported a lower activity of peroxidase enzyme and an increased activity of polyphenol oxidase due to yellow vein mosaic disease of mesta.

Rathi *et al.* (1986) assayed PPO activity in pigeon pea cultivars resistant and susceptible to sterility mosaic virus and reported that inoculation resulted in increased PPO activity in susceptible variety but not in resistant one. Zaidi *et al.* (1992) reported the changes in phenolic content and phenylalanine ammonialyase (PAL) in response to infection by *Carnation etch ring virus*. The results suggested the existence of a positive correlation between the elevated levels of phenolics and phenylalanine ammonialyase with disease resistance. Umamaheswaran (1996) reported that there was progressive increase in peroxidase, polyphenol oxidase and phenylalanine ammonialyase activity in CABMV inoculated and inoculated

susceptible varieties of cowpea. Radhika and Umamaheswaran (2000) reported higher activity of peroxidase, polyphenol oxidase and phenylalanine ammonialyase in resistant variety when compared to susceptible variety of cowpea infected with BICMV. Sindhu (2001) investigated on changes of defence related enzymes *viz.*, peroxidase, polyphenol oxidase and phenylalanine ammonialyase and indicated that there was significant increase in activities of these enzymes in inoculated cowpea plants. Krishnapriya (2015) reported a higher peroxidase, polyphenol oxidase and phenylalanine ammonialyase activity for BlCMV inoculated cowpea plants compared to healthy plants.

Bhatia *et al.* (2004) found that the activities of enzymes superoxide dismutase and peroxidase were increased as against decreased activities of catalase in TMV infected tobacco leaves. The trend was reversed when the leaves were treated with AVP alone. However, the activities of all the three enzymes decreased in TMV plus AVP treated leaves and it was midway between TMV alone and AVP alone treatments.

Verma and Prasad (1984) found that spraying aqueous leaf extract of *Clerodendron aculeatum* prevented infection of *Sunhemp rosette virus* on cluster beans. The resistance thus induced was due to increased activity of catalase, peroxidase and polyphenol oxidase. Verma *et al.* (1996) reported that the pre inoculation application of AVPs challenged with plant viruses in different hosts results in the activation of key enzymes like PO, PPO, PAL, chitinase and glucanase leading to the suppression of viral pathogen. Veena (2007) reported a similar increase in defence related enzymes *viz.*, peroxidase, polyphenol oxidase and phenylalanine ammonialyase in AVP treated cowpea plants challenged with BlCMV compared to that of the healthy and virus inoculated cowpea plants.

*In vitro* raised plantlets of *Feronia limonia* (L.) Swingle when colonized with *P. indica* exhibited an increase in peroxidase activity compared to the control plants (Vyas *et al.*, 2008). Fifteen defence and stress-related genes were reported to be

moderately upregulated in the roots of Arabidopsis when colonized by *P. indica* (Vahabi *et al.*, 2013). Among fourteen days of co-cultivation, a strong difference in the response of defence enzymes were observed before six days and later no remarkable changes were observed in *P. indica* colonized Arabidopsis plants (Vahabi *et al.*, 2015). At the early time point of co-cultivation of *P. indica* with Arabidopsis, stress and defence-related genes were up-regulated thereby appearing to respond to chemical mediators released by the fungus (Vahabi *et al.*, 2015). The anti oxidant enzyme peroxidase was reported to over accumulate in Arabidopsis plants due to the colonization of *P. indica* in its roots (Trivedi *et al.*, 2016).

### 2.6.6. Electrophoretic Analysis of Proteins by SDS-PAGE

Lima *et al.* (1979) reported that by the method of Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE), inclusions of BlCMV consisted a single protein with molecular weight (MW) of 70 kDa whereas, freshly purified BlCMV had a protein with MW of 34 kDa and 2 small proteins with MW of 29 kDa and 27 kDa. Parent and Asselin (1987) described the pattern of proteins that accummulated in the intercellular fluids (IF) of potato leaves on TMV infection and found that these IF proteins contained chitinase, β -1, 3 glucanase and peroxidase. Analysis of purified isolate of BlCMV (BlCMV-AC) from alyce-clover by SDS-PAGE, revealed a major and a minor protein component with relative MW of 34.5 and 31 kDa respectively (Zhao *et al.*, 1991).

SDS-PAGE of extracts from leaves infected with BCMV strains showed that the apparent MW's of the capsid protein of the serotype A and B isolates were 33 and 35 kDa respectively (Vetten *et al.*, 1992). Maurhofer *et al.* (1994) performed polyacrylamide gel electrophoresis of TMV affected *Nicotiana glutinosa* plants which were previously inoculated with *P. fluoroscens* strain CHAO. Several clearly

visible protein bands appeared on the gel. The PR proteins 1a, 1b and 1c were identified together with the β- 1, 3 glucanase and the endochitinase P and Q.

Muthulakshmi and Renukadevi (2001) found that reduction in percentage of infection by *Rice tungro virus* may be due to induction of defence related proteins (PR 1 and PR 2) by application of AVP. Ayisha (2005) performed polyacrylamide gel electrophoresis to analyse the protein profile of cowpea plants under inoculated and uninoculated conditions. The experiment revealed three newly induced virus related proteins in the inoculated cowpea plants compared to healthy control. The new induced proteins obtained were with molecular weight 28 kDa, 15 kDa and 6.2 kDa.

EL-kewey *et al.* (2007) reported that a large MW of 35 kDa was observed when CABMV purified virus preparation was subjected to SDS-PAGE. Bhadramurthy and Bhat (2009) reported that partially purified BCMV subjected to SDS-PAGE, revealed a major band corresponding to 34 kDa. Krishnapriya (2015) reported an increasing trend in the induction of novel proteins in BlCMV inoculated cowpea plants with its maximum values at later stages of observation.

The induction of defence proteins and enzymes by various biotic and abiotic factors were well established in inhibition of viral diseases in plants (Deverall and Dann, 1995; Mali *et al.*, 2000). Veena (2007) reported a decreasing trend in the induction of novel proteins with maximum protein induction for plants treated with biotic and abiotic factors compared to that of healthy and inoculated control.

# *Materials and Methods*

# 3. MATERIALS AND METHODS

## 3.1. MAINTENANCE OF VIRUS AND ENDOPHYTES

### 3.1.1. Symptomatology

Leaves showing typical mosaic and vein banding symptoms were collected from the field and the virus was transmitted to the selected cultivars in insect proof glass house. Symptomatology was studied by observing the development of symptoms in artificially inoculated cowpea and *C. amaranticolor*.

### 3.1.2. Maintenance of Virus

Cowpea variety Sharika (*Vigna unguiculata* var. *sesquipedalis* (L.) Verdcourt) from Department of Plant Breeding and Genetics, College of Agriculture, Vellayani and local lesion host (*Chenopodium amaranticolor*) from Department of Plant Pathology, College of Agriculture, Vellayani were selected for the study. Leaves showing the typical symptoms of vein banding and mosaic were collected from the field and the culture was maintained by repeated transfers on selected cultivars through mechanical transmission. Virus infected cowpea were used as source of virus inoculum. Sap transmission was conducted using 0.1M Sodium phosphate buffer (pH 7.0) (Appendix I). Using pre-chilled mortar and pestle the symptomatic young leaves were homogenized with the Phosphate buffer at the rate of 1 g per 1.5ml of buffer. The homogenate was maintained in an ice box and immediately used for inoculation. Prior to inoculation, the leaves of test plants were uniformly dusted with carborandum powder (600 mesh). Inoculation was done by gently rubbing the cowpea at two leaf stage and *Chenopodium amaranticolor* leaves at 8-10 leaf stage, using forefinger, dipped in the inoculum. After 5 min, the surface was rinsed off with distilled water.

### 3.1.3. Maintenance of Fungal Endophytes

Fungal root endophyte, *Piriformospora indica* and *Fusarium pallidoroseum* from Department of Plant Pathology were maintained in Potato dextrose agar medium (Appendix II) by continuous subculturing. The media was prepared and sterilized by autoclaving at 121$^0$C at pressure about 15 pounds of psi for 20 min. After cooling it was poured into sterile petri dishes of nine centimetre diameter and allowed to solidify. The culture disc of five mm diameter cut out from seven day old culture of fungus was used for sub culturing. The culture disc of the fungus was inoculated into petri dish which was later incubated at room temperature .

## 3.2. DETECTION OF *Cowpea mosaic virus* USING SEROLOGICAL METHOD

The virus isolates from diseased samples were diagnosed using monoclonal and polyclonal antibodies developed specifically against *Black eye cowpea mosaic virus* (BlCMV) and *Cowpea aphid borne mosaic virus* (CABMV) infecting cowpea using Enzyme linked immunosorbent assay (ELISA) and Dot immunobinding Assay (DIBA).

### 3.2.1. Direct Antigen Coating-Enzyme Linked Immunosorbent Assay (DAC-ELISA)

ELISA was carried out to detect the presence of BlCMV/ CABMV in infected cowpea leaves. The procedure described by Huguenot *et al.* (1993) was followed for the detection.

Young infected leaf (1 g) was homogenized in 5 ml of coating buffer (carbonate buffer) containing 2 per cent (w/v) PVP under chilled condition. Healthy plant extract was prepared by using leaves of healthy plants. The homogenate was centrifuged at 500 rpm for 10 min at 4$^0$C ( Hettich zentrifugen). 100 µl of antigen was dispensed in to Nunc immunological plates. The treatments were replicated

thrice and incubated for 1 h at 37$^0$C. The wells were washed with Phosphate buffer Saline-Tween (PBS-T) three times each for duration of 3 min using an ELISA plate washer (pw-40, BIORAD). Blocking was done with 100 µl of 5 per cent spray dried milk (SDM) for 1h at 37$^0$C. After incubation plates were washed with PBS-T as before. Then plates were treated with 100 µl of monoclonal / polyclonal antibodies (Agdia Pvt. Ltd.), at 1: 200 dilutions in PBS-T along with Poly vinyl pyrolidone ovalbumin (PBS-TPO). Three replications were maintained for each treatment and incubated overnight under refrigerated conditions at 4$^0$C. The plates were washed again with PBS-T and then treated with 100 µl of anti-mouse immunoglobulin / antirabbit immunoglobulin (SIGMA-Aldrich) diluted in PBS-TPO and incubated for 2 h at 37$^0$C. Wells were washed with PBS-T in an ELISA washer. The substrate para - nitro phenyl phosphate (*P*-NPP) in diethanolamine buffer (1mg per ml) was added to each well (100 µl per well) and incubated for 10-15 minutes at 37$^0$C. Reaction was stopped by adding 50 µl of 4 per cent Sodium hydroxide. The absorbance was read at 405nm in an ELISA reader (Microplate Reader 680, BIORAD) (Appendix III).

Monoclonal antibodies (MAbs) against BlCMV ( 15E6, 16G5 ) and CABMV( 7A10, 5H5 ) and poyclonal antibody (PAb) against BlCMV (H2) and CABMV (H4) at 1:200 dilutions in PBS-TPO were used for detection. For MAbs, alkaline phosphatase conjugated anti-mouse immunoglobulin (SIGMA-Aldrich) and for PAbs, alkaline phosphatase conjugated antirabbit immunoglobulin (SIGMA-Aldrich) diluted in PBS-TPO (1:10,000 v/v) was used as the secondary conjugate.

### 3.2.2. Dot Immunobinding Assay (DIBA)

DIBA was carried out to detect the presence of BlCMV / CABMV in infected cowpea leaves. Polyclonal antibodies against BlCMV (H2) and CABMV (H4) were used for the study.

Sap was extracted from infected tissue in antigen extraction buffer (1:10 w/v) and filtered through cheese cloth. Expressed sap (0.8 ml) was taken in an eppendorf tube to which 0.4 ml chloroform was added. The mixture was vortexed and centrifuged at 12,000 rpm for 2 min. The clarified sap (upper aqueous layer) was mixed with antigen extraction buffer (1:4 ratio) and vortexed. Nitrocellulose membrane (NCM) in squares of 1 x 1 cm was floated in tris buffer saline (TBS) and air dried. The sample (10 µl) was spotted at the centre of each square and allowed to dry. Treated NCM was immersed in blocking solution with gentle oscillation for 1 h at room temperature. NCM was rinsed in TBS for 10 min and incubated overnight at $4^0$C in crude antiserum diluted in TBS-Spray Dried Milk (SDM). NCM was again rinsed in TBS for 10 min and incubated for 1h at room temperature in secondary antibody (antirabit IgG alkaline phosphatase conjugate diluted in TBS-SDM). After rinsing in TBS for 10 min, NCM was incubated in a solution of Nitro blue tetrazolium (NBT) and Bromo chloro indolyl phosphate (BCIP) at room temperature in the dark for colour development. NCM was rinsed in fixing solution for 10 min after the colour development and then air dried between Whatman filter paper sheets and stored (Appendix IV).

## *3.3.* DEVELOPMENT OF MOLECULAR PROBE(S) FOR THE DETECTION AND DIAGNOSIS OF *COWPEA MOSAIC VIRUS*

Molecular diagnosis via Reverse Transcription – Polymerase chain reaction (RT-PCR) was performed for the detection of the BlCMV infecting cowpea. RNA extraction was done using TRIsoln (Genei Catalogue No. 612106481001730).

### 3.3.1. TRIsol Method for RNA Isolation

#### *3.3.1.1. Sample preparation*

TRIsoln (1 ml) was added to 100 mg of liquid nitrogen ground leaf sample and centrifuged at 12,000 rpm for 10 min at $4^0$C . The cleared supernatant was transferred to a new tube and incubated for 5 min at room temperature. Chloroform (200 µl per 1 ml of TRIsoln) was added to the supernatant and the tube was shaken vigorously for 15 sec. Tube was incubated for 2-3 min at room temperature and centrifuged at 12,000 rpm for 15 min at $4^0$C. The upper, clear aqueous phase of the sample was pipette out for the isolation of RNA.

#### *3.3.1.2. RNA isolation*

To the upper clear phase, 100 per cent Isopropanol (500 µl) was added, mixed gently and incubated for 10 min at room temperature. The mixture was centrifuged at 12,000 rpm for 10 min at $4^0$C.

#### *3.3.1.3. RNA wash*

The supernatant was removed from the tube leaving only the RNA pellet. The pellet was washed with 1 ml of 75 per cent ethanol, vortexed briefly and centrifuged at 7,500 rpm for 5 min at $4^0$C. The wash was discarded and the pellet was air dried for 5 to 10 min.

#### *3.3.1.4. RNA resuspension*

The RNA pellet was resuspended in RNAse free water, followed by incubation in a water bath (55 to $60^0$C for 15 min). RNA was later stored at $-80^0$C.

#### *3.3.1.5. Agarose Gel Electrophoresis for RNA Quality check*

The quality of RNA isolated was checked using agarose gel electrophoresis. An agarose gel of 1.2 per cent was prepared in 1X TAE buffer and ethidium bromide was added (0.5 µl per L). An aliquot of the sample (3 µl) mixed with dye was loaded. The gel was run at 5 V $cm^{-1}$ (Hoefer Powerpack, Germany) for 30 min. The gel was then visualized using BIORAD Molecular Imager (Gel DOC ™ XR+).

### 3.3.2. cDNA Synthesis

The RNA isolated from leaf samples were reverse transcribed to cDNA using Two step AMV RT- PCR kit (GeNei, Catalogue No. 610662400011730).

A master mix was prepared on ice (Appendix V). Master mix was mixed thoroughly by watering for not more than 5 s and was stored on ice. To collect the residual liquid from the walls of the tube, the master mix was centrifuged briefly. To set more than one reaction, appropriate volumes of master mix was dispensed into individual reaction tubes and stored the tubes on ice. Template RNA was added to each tube by vortexing for not more than 5 s and was incubated for 90 min at $42^0$C. The enzyme was inactivated by heating at $85^0$C for 5 min.

### 3.3.3. Primer Designing

Primer designing was based on the nucleotide sequence of the coat protein gene, availed from the National Center for Biotechnology Information (NCBI), using primer 3 software.

### 3.3.4. Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

RT-PCR reaction during the study was performed using the primer pairs designed and also using the primer pairs from the published papers.

#### *3.3.4.1. Primers Used*

| Virus | Direction | Sequence (5'► 3') | Reference |
|---|---|---|---|
| BlCMV-1 | Forward | ATGATGGATGGAGATGAGCA | Designed |
| | Reverse | ATAGACGTCGGCATTAAAGC | |

| | | | |
|---|---|---|---|
| BlCMV-2 | Forward | ATCCAAAACATCGGATCGAG | (Krishnapriya, 2015) |
| | Reverse | TTTTGGTTAACGTCCCTTGC | |
| CABMV-1 | Forward | AATTATGCACCACTTCTCGG | Designed |
| | Reverse | GTCTGACTGGATATATGCGTAC | |
| CABMV-2 | Forward | CGCTCAAACCCATTGTAGAA | (Gillaspie *et al.*, 2001; Damiri *et al.*, 2013) |
| | Reverse | TATTGCTTCCCTTGCTCTTTC | |

### *3.3.4.2. RT-PCR Analysis*

RT-PCR amplification reactions were carried out in a 20 µl reaction volume which contained: 1X Phire PCR buffer (contains 1.5 mM $Mgcl_2$), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl cDNA, 0.2 µl Phire Hostart II DNA polymerase enzyme, 0.1 mg per ml BSA and 3 per cent DMSO, 0.5M Betaine and 5M of forward and reverse primers.

### *3.3.4.3. RT-PCR amplification profile*

RT-PCR amplification profile for BlCMV-1

| | | | |
|---|---|---|---|
| $92^0$ C | - | 2 min | |
| $94^0$ C | - | 30 sec | 35 cycles |
| $50^0$ C | - | 1 min | |
| $72^0$ C | - | 1 min | |
| $72^0$ C | - | 10 min | |

$4\ ^{0}C$ - ∞

RT-PCR amplification profile for BlCMV-2

| | | | |
|---|---|---|---|
| $98\ ^{0}C$ | - | 30 sec | |
| $98\ ^{0}C$ | - | 5 sec | 40 cycles |
| $55\ ^{0}C$ | - | 10 sec | |
| $72\ ^{0}C$ | - | 15 sec | |
| $72\ ^{0}C$ | - | 60 sec | |
| $4\ ^{0}C$ | - | ∞ | |

### *3.3.4.4. Agarose Gel Electrophoresis of RT-PCR Products*

The RT-PCR products were checked in 1.2 per cent agarose gels prepared in 0.5X TAE buffer containing 0.5 µg /ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products, loaded and electrophoresis buffer for about 1-2 h. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator system (Bio-Rad) (Appendix V).

## 3.4. STANDARDIZATION OF CO-CULTIVATION METHOD

Cocultivation of *P. indica* and *F. pallidoroseum* with cowpea and *C. amaranticolor* were standardized for screening it against BlCMV.

### *3.4.1.1. Co-cultivation of Plants and Fungal Endophyte in Plant Nutrient Media (PNM)*

For *in vitro* co-cultivation of the two symbionts, a modified PNM medium (Appendix VI) was used. PNM medium was optimized in such a way that allows

growth of both organisms within the time experiment. Two leaf stage cowpea, *C. amaranticolor*, *P. indica* and *F. pallidoroseum* plugs are used.

In jam bottles fungal plugs of 5 mm diameter from 4 week old fungal PDA plates were placed at the centre of the PNM media. The bottles were sealed with parafilm strips and incubated for 7 days at 22°C. Twelve day old seedlings were transferred to each bottles having fungal mycelia grown for about 4cm in diameter. Later the bottles were incubated at 22°C under continuous illumination. Similarly *C. amaranticolor* was also transferred to each bottles having *P. indica*.

Similarly co-cultivation studies were also conducted for *F. pallidoroseum* with cowpea and *C. amaranticolor*.

### *3.4.1.2. Co-cultivation of Plants and Fungal Endophyte in Vermiculite - Perlite medium*

Planting medium was prepared by mixing vermiculite - perlite in the ratio 3:1 by volume and slightly moistened. Sterilization was done by performing autoclaving (121°C for one hour each) for three consecutive days. Fungal mycelia (*P. indica* and *F. pallidoroseum*) disc of 1 mm size was inoculated to 250 ml of potato dextrose broth and incubated at 28°C for 10 days. The mycelia growth was filtered out using a strainer and washed twice with sterile water. Mycelia was added to the sterile planting medium at the rate of 1% (w/v), mixed properly and filled in portray cavities. Seeds of local lesion host *C. amaranticolor* and cowpea variety Sharika was surface sterilized with 1% sodium hypochlorite for five min. followed by three washing with sterile water and blot dried with blotting paper. Seeds were sown to the planting media containing fungus. Plants were maintained in a growth chamber and watered twice a day with sterile water.

### *3.4.1.3. Root Colonization by Fungal Root Endophytes*

Roots were collected from cowpea and *C. amaranticolor* seedlings treated with *P. indica* and *F.pallidoroseum*. Roots were carefully separated from the plant,

washed thoroughly with tap water to get rid of the planting media. Roots were cut into small pieces of approximately of one cm. Root bits were transferred to a small beaker having 5 ml of 10% KOH and boiled for 5 min. KOH solution was drained out from the beaker and three washing with tap water was given. Then the root bits were soaked in 2% HCl for 5 min. Root bits were then removed from the acid and transferred to lactophenol trypan blue for 10 min. for staining. The root bits were viewed under a microscope and checked for presence of chlamydospores in each root bit. The percentage root colonization was found out by the following formula described by Krishnaveni *et al.* (2015),

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

***3.4.1.4. Age of the Seedlings Study***

Vermiculite - perlite in the ratio 3:1 by volume slightly moistened by water and sterilized using autoclave ($121^0$C for one h each) for three consecutive days were used as planting medium. Seeds of cowpea variety Sharika was surface sterilized with 1% sodium hypochlorite for five minutes followed by three washing with sterile water and blot dried with blotting paper. Plants were maintained in a growth chamber and watered twice a day with sterile water. Fifth, seventh, tenth and fifteen days old seedlings were transferred to planting media added with *P. indica*. Biometric observations were conducted at seven, fifteen and twenty days of co-cultivation.

Colonization pattern was studied by staining the roots as mentioned above using lactophenol trypan blue and observing it under the compound microscope at different focal length. Colonization efficiency was calculated by using the above mentioned equation for percentage root colonization. Number of secondary and tertiary roots and leaf number was counted as such while root hairs per unit area and leaf area was calculated using graphical method.

## 3.5. MANAGEMENT OF BICMV USING BOTANICALS AND FUNGAL ROOT ENDOPHYTES

Plants possessing antiviral proteins were screened for their efficiency against BICMV. Leaf extracts of such plants were prepared at 1%, 2.5% and 5% concentrations. The plants mentioned below are widely used in ayurvedic medicines against various human diseases. The following are the list of botanicals having antiviral proteins.

**Table 1. Botanicals used for bioassay in local lesion host**

| Treatment No. | Botanicals having antiviral proteins | Plant part used | Stage of plant |
|---|---|---|---|
| $T_1$ | *Boerhaavia diffusa* L. | Leaf | Flowering |
| $T_2$ | *Phyllanthus niruri* L. | Leaf | Fruiting |
| $T_3$ | *Calotropis gigantean* L. | Leaf | Flowering |
| $T_4$ | *Psidium guajava* L. | Leaf | Fruiting |
| $T_5$ | *Zingiber officinale* L. | Rhizome | Vegetative |
| $T_6$ | *Aloe vera* L. | Leaf | Vegetative |
| $T_7$ | *Euphorbia hirta* L. | Leaf | Flowering |

*Piriformospora indica* was the fungal endophyte used for the study.

### 3.5.1. Bioassay of Botanicals in Local Lesion Host

The efficacy of the botanicals mentioned above were evaluated in the local lesion host of the virus, *C. amaranticolor*. Pre-inoculation, simultaneous inoculation and post-inoculation treatments were done. Experiment was laid out in CRD with three replications.

In pre-inoculation, above mentioned botanicals were sprayed 24 h prior to mechanical inoculation of virus. For mechanical transmission of virus, sap was extracted from young leaves showing severe mosaic symptoms. One gram of the leaf tissue was homogenized with 1.5 ml of ice cold 0.01M phosphate buffer (pH 7.2) using a chilled mortar and pestle. The homogenate was strained through a thin layer of cotton. The supernatant was maintained in an icebox and immediately used for inoculation. Inoculation was done on the leaves of local lesion host, *C. amaranticolor*. Plants at 8-10 leaf stage were chosen. Leaves at the middle portion of the plant (Fourth to eighth leaves) were inoculated. Prior to inoculation leaves were uniformly dusted with carborandum powder. Test plants were inoculated by gently rubbing on the upper surface of the fully opened leaves. The surface was rinsed off after 5 min. with distilled water using a wash bottle.

In simultaneous inoculation, above mentioned botanicals and virus inoculum were mixed together and applied. Botanicals were sprayed 24 h after inoculation of virus, in post-inoculation. The plants were kept for 5-6 days for the development of symptoms. Control leaves were maintained without any treatment.

Local lesions were recorded for evaluating the efficiency. From this per cent inhibition was calculated based on the formula.

$$\text{Percent inhibition} = \frac{C-T}{C} \times 100$$

C=Number of lesions on control leaves

T= Number of lesions on treated leaves

### 3.5.2. Evaluation of the Endophyte Primed or AVP-treated Cowpea Seedlings against BlCMV

A pot culture experiment was laid out in CRD with three replications. Treatments of botanical which showed high inhibition percentage in local lesion assay were selected for the bioassay in cowpea plants. Plant extract of *P. niruri* (1 per cent) and fungal root endophyte *P. indica* (1 per cent) were used for evaluation. Pre and post inoculation treatments were done as that of previous experiment at primary leaf stage.

In case of *P. indica* seeds were sown into the fungus inoculated potting mixture and twelve days old seedlings were inoculated with the virus for pre inoculation studies. In post inoculation, virus was inoculated at two leaf stage and was later transplanted to fungus inoculated potting mixture. Control plant was also maintained.

Effects of materials on expression of symptoms were recorded. Based on the severity of symptoms, vulnerability index was calculated. This was in accordance with the 0-5 scale 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 rating, Vulnerability Index (VI) was calculated using the following equation,

$$VI = \frac{(0n_1 + 1n_2 + 2n_2 + 3n_3 + 4n_4 + 5n_5) \times 100}{n_t (n_c - 1)}$$

| | | |
|---|---|---|
| VI | = | Vulnerability Index |
| $n_0, n_1 \ldots\ldots n_5$ | = | number of plants in the category 0,1 2,3,4,5 |
| $n_t$ | = | Total number of plants |
| $n_c$ | = | Total number of categories |

### 3.5.3. Biochemical Changes of Host Pathogen Interaction

Biochemical analysis of healthy, diseased and treated plants were carried out. Cowpea variety Sharika was selected for the study. Seeds were sown and mechanically inoculated at primary leaf stage. Treatments were done as pre and post application. Samples were taken at five, ten, fifteen and thirty days after inoculation.

Biochemical analysis was conducted to estimate the changes in total soluble protein, total sugar, reducing sugar and phenol content. Analysis of defence related enzymes such as peroxidase, polyphenol oxidase, and phenylalanine ammonialyase were also carried out. Protein profile study was performed using SDS-PAGE.

#### *3.5.3.1. Estimation of Protein*

Total soluble protein content was estimated as per the procedure described by Bradford (1976). One gram of leaf sample was homogenized in 10 ml of 0.1 M sodium acetate buffer (pH 4.7) (Appendix VII) and centrifuged at 5000 rpm for 15 min at $4^0$C. The supernatant was collected for estimation of soluble protein. The reaction mixture consisted of 0.5 ml enzyme extract, 0.5 ml distilled water and 5

ml of diluted (5 times) dye solution. Blank was prepared with 1 ml distilled water and 5 ml of diluted (5 times) dye solution (Appendix VII). The absorbance was read at 595 nm in a spectrophotometer (Eppendorf Bio Spectrometer) against reagent blank. Bovine serum albumin was used as the protein standard. The protein content was expressed as microgram albumin equivalent of soluble protein per gram on fresh weight basis.

### *3.5.3.2. Estimation of Total Sugars*

Total sugar content was estimated by Anthrone method (Hedge and Hofreiter, 1962). Samples of 100 mg each were weighed out and hydrolyzed with 5 ml of 2.5 N hydrochloric acid (HCl) in a boiling water bath. The hydrolyzate was neutralized with solid sodium carbonate until the effervescence ceased. The volume was made up to 100 ml and centrifuged at 5000 rpm for 15 min. From the supernatant 0.5 ml aliquot was taken and made up to one ml by adding distilled water. To this 4 ml anthrone reagent was added and heated for eight minutes in a boiling water bath. This was cooled rapidly and absorbance was measured at 630 nm in a spectrophotometer (Eppendorf Bio Spectrometer). Blank was prepared with 1 ml distilled water and 4 ml anthrone reagent (Appendix VIII). Amount of carbohydrate present was calculated from standard graph prepared using glucose and expressed in terms of milligrams of glucose equivalent per gram of leaf tissue on fresh weight basis.

### *3.5.3.3. Estimation of Reducing Sugars*

Reducing sugar content was estimated by dinitrosalicylic acid method. One hundred milligram leaf sample was ground in 10 ml of 80 percent ethanol twice with 5 ml each time and centrifuged at 5000 rpm for 15 min. The supernatant was collected and evaporated by keeping it on a water bath at $80^0$C. The sugars were dissolved in 10 ml distilled water. An aliquot of 0.5-3 ml was pipetted out in test

tubes and volume was equalized to three ml with distilled water in all the tubes. To this 3 ml of dinitrosalicylic acid reagent (DNS) (Appendix IX) was added. Contents were heated in a boiling water bath for 5 min and to this 1 ml of 40 percent rochelle salt solution was added. After cooling the absorbance was measured at 510 nm in a spectrophotometer (Eppendorf Bio Spectrometer) against reagent blank. Amount of reducing sugar was calculated from standard graph prepared using glucose and expressed in terms of milligrams of glucose equivalent per gram of leaf tissue on fresh weight basis.

#### *3.5.3.4. Estimation of Phenol*

The phenol content was estimated following the procedure described by Bray and Thorpe (1954). One gram leaf sample was ground in 10 ml of 80 per cent ethanol. The homogenate was, centrifuged at 10000 rpm for 20 min and the supernatant was saved. The residue was extracted with five times the volume of 80 per cent ethanol and again centrifuged. The supernatant was saved and evaporated to dryness. The residue was dissolved in 5 ml distilled water. An aliquot of 0.3 ml was pipetted out and made up to 3 ml with distilled water. Folin - Ciocalteau reagent (0.5 ml) and 2 ml of 20 per cent sodium carbonate solution was added to each tube after three minutes. This was mixed thoroughly and kept in boiling water for one min. After cooling absorbance was measured at 650 nm in a spectrophotometer (Eppendorf Bio Spectrometer) against reagent blank. Blank was prepared with 3 ml distilled water, 0.5 ml Folin - Ciocalteau reagent and 2 ml of 20 per cent sodium carbonate solution. Standard curve was prepared using different concentrations of catechol. The phenol content was expressed in catechol equivalents as microgram per gram leaf tissue on fresh weight basis.

#### *3.5.3.5. Estimation of Defence Related Enzymes*

*3.5.3.5.1. Estimation of Peroxidase (PO)*

Peroxidase activity was determined as per the procedure described by Srivastava (1987). Leaf sample of 200 mg was homogenized in one ml of 0.1 M sodium phosphate buffer (pH 6.5) (Appendix X) to which a pinch of polyvinyl pyrrolidone (PVP) was added. The homogenization was done at $4^0$C using a mortar and pestle. The homogenate was filtered through a muslin cloth and centrifuged at 5000 rpm for 15 min at $4^0$C. The supernatant was used as the enzyme extract for the assay of PO activity.

The reaction mixture consisting of 1 ml 0.05 M pyrogallol and 50 µl of enzyme extract was taken in both reference and sample cuvettes mixed well and kept in a spectrophotometer ( Eppendorf Bio Spectrometer). The reading was adjusted to zero at 420 nm. The enzyme reaction was started by adding one ml of one per cent hydrogen peroxide ($H_2O_2$) (v/v) into sample cuvettes and change in absorbance was measured at 30 seconds interval.

*3.5.3.5.2. Estimation of Polyphenol Oxidase (PPO)*

Polyphenol oxidase activity was determined as per the procedure given by Mayer *et al.* (1965). The enzyme extract was prepared as per the procedure given for the estimation of peroxidase.

The reaction mixture contained one ml of 0.1 M sodium phosphate buffer (pH 6.5) (Appendix X)and 50 µl of enzyme extract. The reaction was initiated after adding one ml of 0.01 M catechol. The observations were recorded in a spectrophotometer (Eppendorf Bio Spectrometer). The change in absorbance was recorded at 495 nm and PPO activity was expressed as change in the absorbance of the reaction mixture per minute per gram on fresh weight basis.

*3.5.3.5.3. Estimation of Phenylalanine Ammonialyase (PAL)*

PAL activity was analysed based on the procedure described by Dickerson *et al.* (1984). The enzyme extract was prepared by homogenizing one gram leaf sample in 5 ml of 0.1 M sodium borate buffer (pH 8.8) (Appendix XI) containing a pinch of PVP using chilled mortar and pestle. The homogenate was centrifuged at 10000 rpm for 10 minutes at $4^0$C. The supernatant was used for the assay of PAL activity. The reaction mixture contained 3 ml of 0.1 M sodium borate buffer (pH 8.8), 0.2 ml enzyme extract and 0.1 ml of 12 mM *l*-phenylalanine prepared in the same buffer. The blank contained 3 ml of 0.1 M sodium borate buffer (pH 8.8) and 0.1 ml of 12 mM *l*-phenylalanine prepared in the same buffer. The reaction mixture and blank was incubated at $40^0$C for 30 min and reaction was stopped by adding 0.2 ml of 3 N hydrochloric acid. The absorbance was read at 290 nm in a spectrophotometer (Eppendorf Bio Spectrometer). PAL activity was expressed as micrograms of cinnamic acid produced per min per gram on fresh weight basis.

***3.5.3.5.4. Electrophoretic Analysis of Proteins by SDS-PAGE***

Electrophoretic separation of soluble protein of cowpea leaves were carried out as per the procedure described by Laemelli (1970). Leaf sample of healthy, diseased and treated plants were taken for analysis.

Five hundred milligram each of healthy infected and treated leaf samples were homogenized in 200 µl of cold denaturing solution (Appendix XII) at $4^0$C. The supernatant was mixed with chilled acetone in the ratio 1:4 and the protein was allowed to precipitate by keeping the mixture at $4^0$C for 30 min. The sample was centrifuged at 5000 rpm for 15 min at $4^0$C. The precipitate was resuspended in 20 µl of denaturing solution and vortexed. The homogenate was centrifuged at 5000 rpm for 15 min. The supernatant was mixed with equal volume of sample buffer and kept in a boiling water bath for 3 min. These samples were used for SDS-PAGE. Ten µl of medium range molecular weight markers (Genei, Bangalore) mixed in 10 µl of

sample buffer were also loaded. The protein concentration was adjusted in each sample to strength of 100 μg of protein following Bradford method. The water layered over the separating gel was removed and washed with a little electrode buffer and then the stacking gel was poured over the polymerized separating gel, after keeping the comb in position.

After polymerization the samples were loaded into the wells. The electrophoresis was performed at 100 V till the tracking dye reached the separating gel. Then the voltage was increased in 200 V and continued till the dye reached the bottom of the gel. The gel was removed immediately after electrophoresis between the glass plates and incubated in the staining solution for overnight with uniform shaking. Then the gel was transferred to the destaining solution (Appendix VII). The protein appeared as bands and the gel was then visualized and photographed using BIORAD Molecular Imager (Gel DOC ™ XR+). The molecular weights of the new and induced polypeptides were calculated from the standard graph prepared for a 12 per cent gel using standard markers.

# *Results*

# 4. RESULTS

The present study on "Exploration of natural products from botanicals and fungal root endophytes for the management of *Cowpea mosaic virus*" was conducted during the period of 2014-2015 at the Department of Plant Pathology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala, to develop serological and molecular tools for the early detection of *Cowpea mosaic virus*; and its management using natural products from botanicals and fungal root endophytes. The results obtained from the experiments are summarized below:

## 4.1. MAINTENANCE OF VIRUS AND ENDOPHYTES

### 4.1.1. Maintenance of Virus

Sap transmission of leaves showing typical symptoms of mosaic and vein banding were done using 0.1 M Sodium phosphate buffer (pH 7.0). Thus by repeated transfers through mechanical inoculation, *Cowpea mosaic virus* was maintained in *Chenopodium amaranticolor* and cowpea variety Sharika.

### 4.1.2. Symptomatology

Symptoms were expressed 7 days after inoculation (DAI) in mechanically inoculated cowpea cultivar, Sharika. On the newly emerged trifoliate leaves, vein clearing was observed as initial symptoms. Later dark green and light green mottling on the leaves was observed. As the infection progressed, typical symptoms like mosaic (Plate 1a) and vein banding (Plate 1b) were noted which was followed by leaf distortion (Plate 1c). Severe stunting of plants was also noted along with reduced pod production. In *C. amaranticolor* symptoms were expressed 5 days after inoculation (DAI). On the inoculated leaves, localized yellow chlorotic lesions were observed (Plate 2).

**Plate 1. Symptomatology on cowpea**

**Plate 1a. Mosaic**

**Plate 1b. Vein banding**

**Plate 1c. Leaf deformation**

**Plate 2. *Chenopodium amaranticolor* on inoculation with virus**

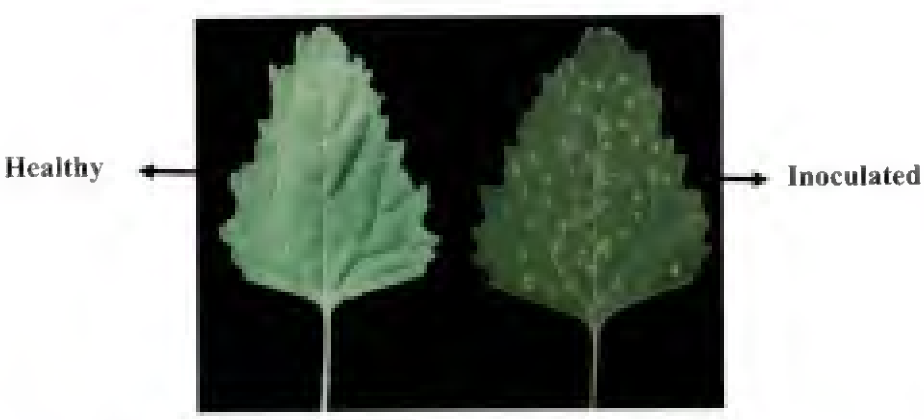



**Plate 3. Maintenance of fungal root endophytes**

Plate 3a. *Piriformospora indica* (PDA medium)

Plate 3b. *Fusarium pallidoroseum* (PDA medium)

### 4.1.3. Maintenance of Fungal Endophytes

Fungal root endophytes *Piriformospora indica* and *Fusarium pallidoroseum* were maintained by continuous subculturing in Potato dextrose agar medium. The *P. indica* produced nine cm radial growth after nine days of inoculation (Plate 3a) whereas *F. pallidoroseum* produced nine centimetre radial growth after five days of inoculation (Plate 3b) in petri dishes.

## 4.2. DETECTION OF *Cowpea mosaic virus* USING SEROLOGICAL METHOD

### 4.2.1. Enzyme Linked Immunosorbent Assay (ELISA)

Direct antigen coating Enzyme linked immunosorbent assay (DAC-ELISA) was used to detect the virus causing mosaic disease in cowpea in kerala. The absorbance was measured at 405 nm in an ELISA reader (BIO-RAD Microplate Reader 680). The antigen source was the crude sap from infected cowpea leaf tissue. Polyclonal and monoclonal antibodies specific to BlCMV and CABMV were used for the study. Results are summarized in Table 2 and 3.

The polyclonal antibody (H2) against BlCMV and polyclonal antibody (H4) against CABMV gave a positive reaction in samples collected from symptomatic plants. The virus infected cowpea leaf extracts gave positive reaction towards BlCMV polyclonal antibody (H2) with an absorbance of 0.242 and polyclonal antibody (H4) with an absorbance value of 0.260.

Two monoclonal antibodies (15E6, 16G5) against BlCMV gave a positive reaction in samples collected from symptomatic plants while it did not react with the monoclonal antibodies (7A10, 5H5) of CABMV. Monoclonal antibodies (15E6, 16G5) of BlCMV gave positive results in infected cowpea plants with an absorbance of 0.387 and 0.519 respectively (Plate 4).

**Table 2. Reaction of polyclonal antibodies against BlCMV / CABMV in infected cowpea plants**

| Polyclonal antibodies | *Absorbance at 405 nm | | Reaction |
|---|---|---|---|
| | Healthy | Infected | |
| H2 (BlCMV) | 0.012±0.003 | 0.242±0.070 | + |
| H4 (CABMV) | 0.010±0.025 | 0.206±0.107 | + |

*Mean of three replications

**Table3. Reaction of monoclonal antibodies against BlCMV / CABMV in infected cowpea plants**

| Monoclonal antibodies | *Absorbance at 405 nm | | Reaction |
|---|---|---|---|
| | Healthy | Infected | |
| 15E6 (BlCMV) | 0.009±0.061 | 0.387±0.056 | + |
| 16G5 (BlCMV) | 0.010±0.011 | 0.519±0.046 | + |
| 7A10 (CABMV) | 0.013±0.004 | 0.007±0.001 | - |
| 5H5 (CABMV) | 0.015±0.005 | 0.006±0.002 | - |

*Mean of three replications

+ Presence of virus

- Absence of virus

**Plate 4. Reaction of BlCMV and CABMV in DAC-ELISA**


| | |
|---|---|
| **Healthy : 2, 3, 4** | **Buffer : 10, 11** |
| **Positive reaction to H2: B6, B7 and B8** | **Positive reaction to H4: E6, E7 and E8** |
| **Positive reaction to 15E6: C6, C7 and C8** | **Negative reaction to 7A10 : F6, F7, F8** |
| **Positive reaction to 16G5: D6, D7 and D8** | **Negative reaction to 5H5 : G6, G7, G8** |

**Plate 5: Reaction of polyclonal antibodies against BlCMV/CABMV in DIBA**

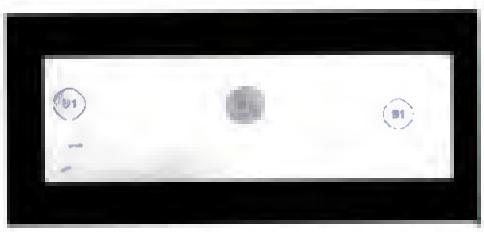


**Plate 5a. Positive reaction against BlCMV : H2**

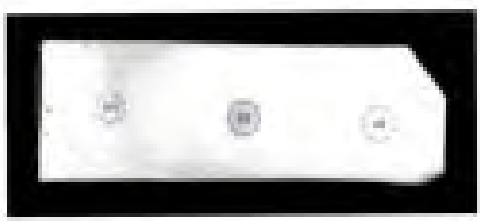


**Plate 5b. Positive reaction against CABMV : H4**

**U1: Healthy** **U2: Diseased** **B: Buffer**

### 4.2.2. Dot Immunobinding Assay (DIBA)

DIBA was conducted to detect the presence of the virus causing mosaic disease in cowpea by using the polyclonal antiserum against BlCMV and CABMV i.e, H2 and H4 respectively. It was observed that, antiserum against both BlCMV (Plate 5a) and CABMV (Plate 5b) gave positive reaction. The infected leaf samples showed definite purple coloured spots on nitrocellulose membrane indicating positive reaction, which was absent in healthy and buffer check which was colourless. Mean value of intensity was calculated using BIORAD Molecular Imager (Gel DOC ™ XR+) and was found to be higher in diseased samples than healthy and buffer (Table 4 and 5).

## 4.3. DEVELOPMENT OF MOLECULAR PROBE(S) FOR THE DETECTION AND DIAGNOSIS OF *Cowpea mosaic virus*

Molecular diagnosis using RT-PCR, followed by gel electrophoresis was performed for the specific detection of the virus causing mosaic disease in cowpea. RNA was isolated from the symptomatic leaves using TRIsoln method (Plate 6) and primers specifically designed based on the nucleotide sequence of coat protein region of the BlCMV and CABMV along with the primers already published were tested for the presence of the virus. There was no amplification with CABMV specific primers. An amplicon of size ~ 425 bp, was obtained from the cDNA reverse transcribed from the infected cowpea leaf sample using the BlCMV specific primer designed and an amplicon of size ~ 110 bp, was obtained using the BlCMV specific primer from the already published paper repectively. Results were observed in Biorad gel documentation system. RT-PCR results confirmed that, the mosaic disease of cowpea in Kerala was caused by the *Black eye cowpea mosaic virus* (BlCMV).

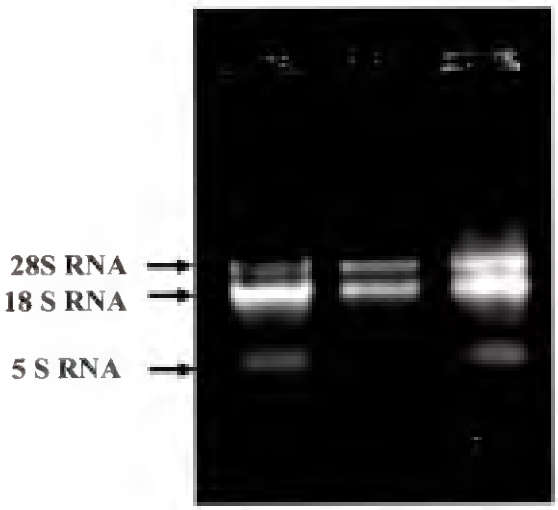
**Table 4 : Reaction of polyclonal antibodies against BlCMV in infected cowpea plants in nitrocellulose membrane**

| Sl.No. | Label | Type | Volume (Int) | Adj. Vol. (Int) | Mean Bkgd. (Int) | Min Value (Int) | Max Value (Int) | Mean Value (Int) | Area (mm2) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | U1 | Healthy | 1,135,253 | 353,870 | 1,056.4 | 628 | 2,753 | 958.4 | 9.4 |
| 2 | U2 | Diseased | 5,757,886 | 729,069 | 2,503.1 | 935 | 3,276 | 2,866.0 | 9.4 |
| 3 | U3 | Background | 1,693,142 | 380,5 91 | 1,231.6 | 657 | 2,648 | 843.1 | 9.4 |

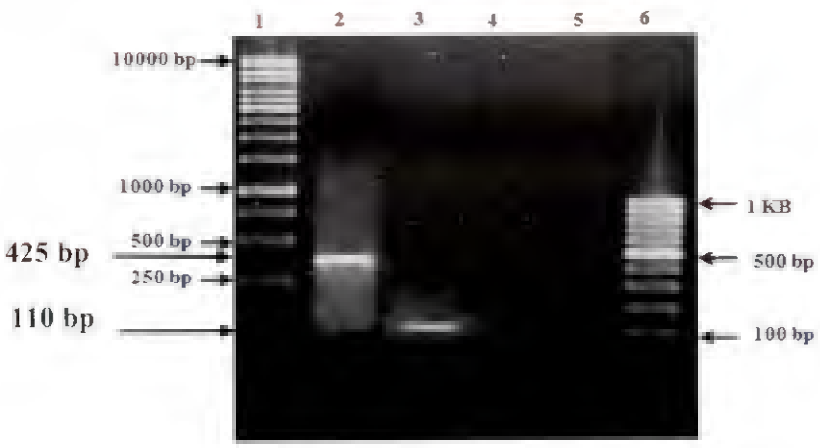
**Table 5 : Reaction of polyclonal antibodies against CABMV in infected cowpea plants in nitrocellulose membrane**

| Sl.No. | Label | Type | Volume (Int) | Adj. Vol. (Int) | Mean Bkgd. (Int) | Min. value (Int) | Max. value (Int) | Mean value | Area (mm2) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | U1 | Healthy | 2,422,909 | 406,532 | 801.1 | 669 | 1,441 | 962.6 | 9.4 |
| 2 | U2 | Diseased | 3,263,805 | 776,528 | 1238.1 | 894 | 2,122 | 1,624.6 | 9.4 |
| 3 | B1 | Background | 2,284,385 | 175,222 | 910.3 | 681 | 1,696 | 985.9 | 9.4 |

**P late 6. Molecular diagnosis of BlCMV**




**Total RNA**




**1-1kb DNA ladder 2- BlCMV (designed primer) 3- BlCMV (published primer)**

**4- CABMV (designed primer) 5- CABMV (published primer) 6- 100 bp DNA ladder**

## 4.4. STANDARDIZATION OF CO-CULTIVATION METHOD

### 4.4.1. Co-cultivation of Plants and Fungal Endophyte in Plant Nutrient Media (PNM)

Co-cultivation of two leaf stage cowpea with four weeks old *P. indica* and *F. pallidoroseum* were conducted in jam bottles. Two leaf stage cowpea on transfer to PNM (Appendix VI) media containing fungus showed decay of cowpea plants. So the standardization of co-cultivation of cowpea with both the fungus were not able to be conducted in Plant nutrient media.

Co-cultivation of *Chenopodium amaranticolor* with *P. indica* and *F. pallidoroseum* were also conducted in jam bottles. No fungal colonization was observed within the roots after co-cultivation.

### 4.4.2. Co-cultivation of Plants and Fungal Endophytes in Vermiculite - Perlite medium

Co-cultivation of cowpea and *Chenopodium amaranticolor* with *Fusarium pallidoroseum* mixed with vermiculite - perlite (3:1) medium at the rate of 1% (w/v) were conducted in protrays. Roots were observed each day after co-cultivation for colonization and percentage colonization was recorded. But no colonization was observed during the co-cultivation studies of *F. pallidoroseum* with both cowpea and *C. amaranticolor*.

Co-cultivation of cowpea and *C. amaranticolor* with *P. indica* mixed with vermiculite - perlite (3:1) medium at the rate of 1% (w/v) were also conducted in protrays. After each day of co-cultivation, roots were observed for colonization. In cowpea, after 7 days of co-cultivation with *P. indica* chlamydospores were observed on the surface of the roots and no colonization was observed inside the roots (Plate 7). But colonization of fungus was observed within the roots after 14 days of

**Plate 7. Chlamydospores of *P. indica* on the root surface of cowpea after 7 days of co-cultivation**

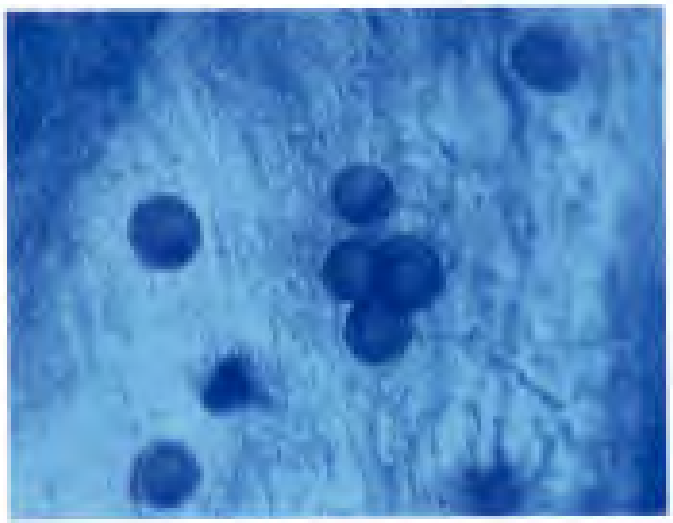
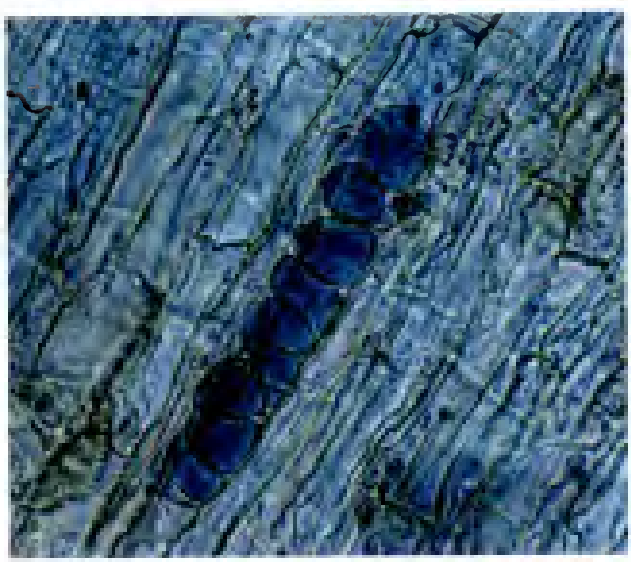


**Plate 8. Colonization of *P. indica* within the roots of cowpea after 14 days of co-cultivation**



co-cultivation of cowpea with *P. indica* (Plate 8). Percentage root colonization was calculated and was observed as 40.7 per cent (Table 6). In *C. amaranticolor,* no colonization was observed within the roots during the co-cultivation studies.

**4.4.3. Age of the Seedlings Study**

Age of the seedlings study for cowpea with *Piriformospora indica* were conducted in protrays. Biometric observations were carried out for the co-cultivation studies.

Colonization pattern was studied and found that five and seven days old seedlings after seven days of co-cultivation showed chlamydospores on the root surface and colonization was observed within the roots after fifteen and twenty days of co-cultivation (Plate 9 and 10) . In case of ten and fifteen days old seedlings after seven days of co-cultivation no colonization was observed whereas, chlamydospores were observed on the surface of the roots after fifteen days of co-cultivation and colonization was observed after twenty days of co-cultivation (Plate 11 and 12). The study revealed that the decrease in age of the seedlings increases the chances of colonization.

Study on the efficiency of colonization revealed that efficiency increases with the decrease in the age of the seedling. Highest efficiency was observed for five days old seedlings at twenty days after co-cultivation. Five days old seedlings recorded an efficiency of 37.5 per cent and 42.3 per cent after fifteen and twenty days of co-cultivation respectively whereas 34.61 per cent and 40.62 per cent efficiency were recorded for seven days old seedlings after fifteen and twenty days of co-cultivation respectively. An efficiency of 32.43 per cent and 22.85 per cent were observed for ten and fifteen days old seedlings respectively after twenty days of co-cultivation (Table 7).

In case of root biomass, maximum percentage of increase (77.77 per cent) was observed for five days old seedlings at twenty days after co-cultivation followed by

**Table 6. Percentage colonization after Co-cultivation of Plants and Fungal Endophytes in Soil**

| Colonization per cent | | |
|---|---|---|
| Crop | *Piriformospora indica* | *Fusarium pallidoroseum* |
| *Chenopodium amaranticolor* | - | - |
| *Vigna unguiculata* | 40.7 | - |

**Table 7. Impact of age of seedlings on colonization efficiency by *P. indica* in cowpea**

| Efficiency (%) | | | |
|---|---|---|---|
| **Treatments** | **7 DAC** | **15 DAC** | **20 DAC** |
| 5 day old seedlings | - | $37.5^{a}$ | $42.30^{a}$ |
| 7 day old seedlings | - | $34.61^{b}$ | $40.62^{b}$ |
| 10 day old seedlings | - | - | $32.43^{c}$ |
| 15 day old seedlings | - | - | $22.85^{d}$ |
| | - | **t(0.05) – (2.776)** | **CD(0.05) - 0.058** |

**DAC – Days after co cultivation**

**Plate 9. Colonization pattern of five days old seedlings**

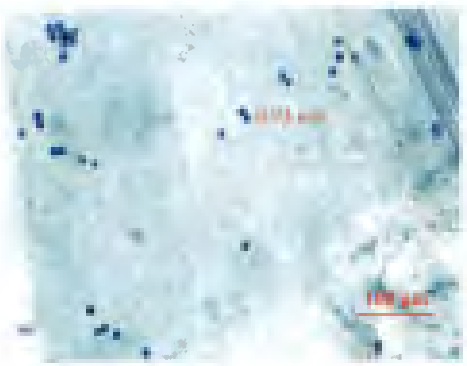


**Plate 9a. Chlamydospores on root surface after seven days of co-cultivation**

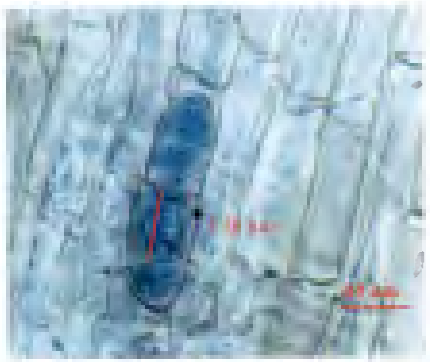


**Plate 9b. Colonization pattern after fifteen days of co-cultivation**

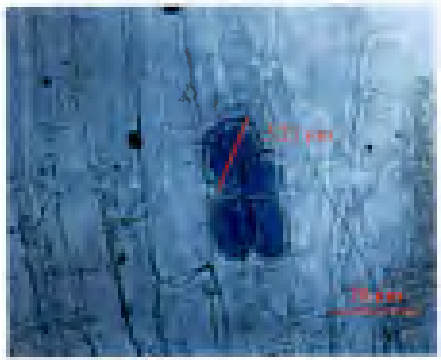


**Plate 9c. Colonization pattern after twenty days of co-cultivation**

**Plate 10. Colonization pattern of seven days old seedlings**

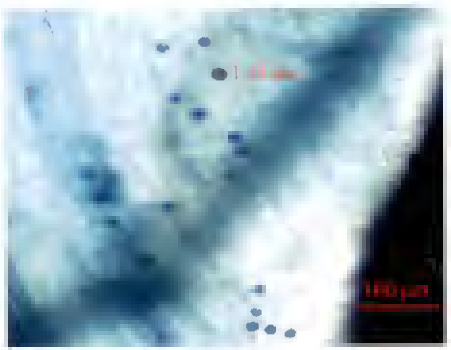


**Plate 10a. Chlamydospores on root surface after seven days of co-cultivation**

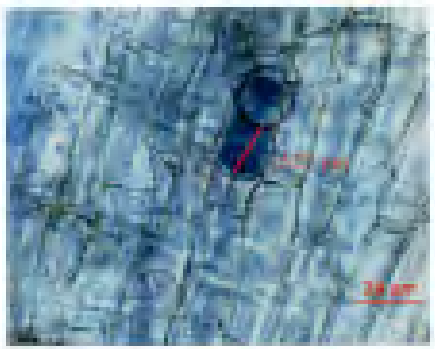


**Plate 10b. Colonization pattern after fifteen days of co-cultivation**

**Plate 10c. Colonization pattern after twenty days of co-cultivation**

**Plate 11. Colonization pattern of ten days old seedlings**

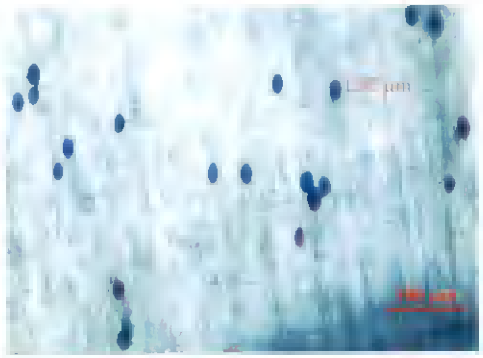


**Plate 11a. Chlamydospores on root surface after seven days of co-cultivation**

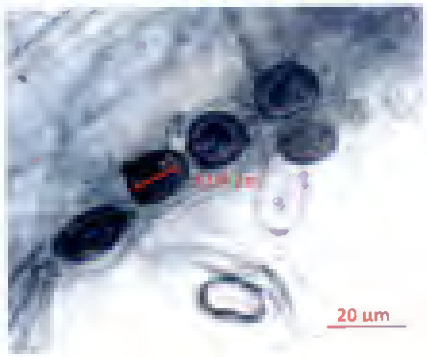



**Plate 11b. Chlamydospores on root surface after fifteen days of co-cultivation**

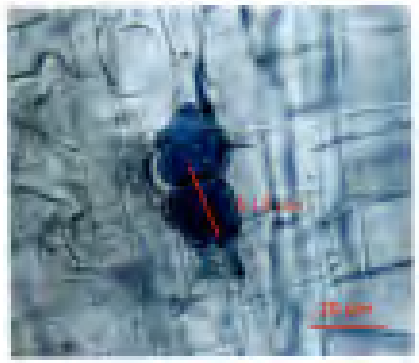


**Plate 11c. Colonization pattern after twenty days of co-cultivation**

**Plate 12. Colonization pattern of fifteen days old seedlings**

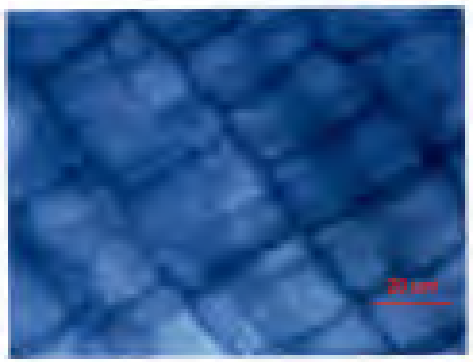


**Plate 12a. No colonization after seven days of co-cultivation**

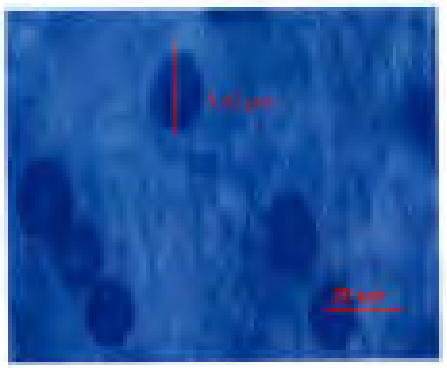


**Plate 12b. Chlamydospores on root surface after fifteen days of co-cultivation**

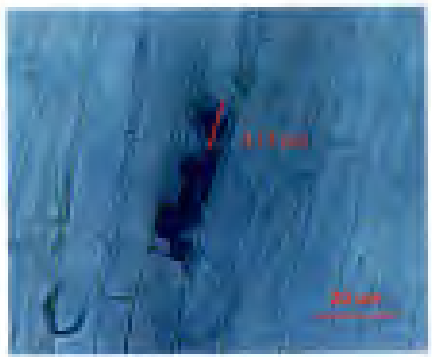


**Plate 12c. Colonization pattern after twenty days of co-cultivation**

seven days old seedlings at twenty days after co-cultivation. Lowest per cent increase in root biomass (6.94 per cent) was observed for fifteen days old seedlings at seven days after co-cultivation (Table 8).

Percentage increase in shoot biomass ranges from 9.09 per cent to 80.68 per cent (Table 9). Highest percentage increase (80.68) in shoot biomass was observed for seven days old seedlings at twenty days of co-cultivation followed by five days old seedlings at twenty days of co-cultivation. Fifteen days old seedlings at seven days of co-cultivation exhibited the lowest shoot biomass.

*P. indica* treated cowpea plants showed an increase in the number of secondary roots than the control plants. Per cent increase was found to be highest (73.54) in five days old seedlings at twenty days of co-cultivation followed by seven days old seedlings (71.36 ) at twenty days of co-cultivation and lowest (11.36) in fifteen days old seedlings at seven days after co-cultivation (Table 10).

A drastic increase in the number of tertiary roots was observed in the treated cowpea plants compared to the control plants. Percentage increase in number of tertiary roots ranges from 11.97 per cent for fifteen days old seedlings at seven days of co-cultivation to 91.46 per cent for five days old seedlings at twenty days of co-cultivation (Table 11).

Root hairs per unit area was calculated using graphical method and the per cent increase ranges from 9.90 per cent to 75.42 per cent. Seven days old seedlings at twenty days of co-cultivation exhibited the highest number of root hairs per unit area followed by five days old seedlings at twenty days of co-cultivation (Table 12).

In case of observations on number of leaves, maximum per cent increase (42.01 per cent) was observed for five days old seedlings at twenty days after co-cultivation and the least value (6.54 per cent) was observed for fifteen days old seedlings at twenty days after co-cultivation (Table 13).

**Table 8. Changes in root biomass with *P. indica* colonization**

| Root biomass (g/plant) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Treatments** | **7 DAC** | | | **15 DAC** | | | **20 DAC** | | |
| | **Control** | **Treated** | **% increase** | **Control** | **Treated** | **% increase** | **Control** | **Treated** | **% increase** |
| 5 day old seedlings | 0.13 | 0.16 | 23.07[a] | 0.26 | 0.41 | 57.69[a] | 0.36 | 0.64 | 77.77[a] |
| 7 day old seedlings | 0.24 | 0.29 | 20.83[b] | 0.41 | 0.62 | 51.21[b] | 0.59 | 1.03 | 76.27[b] |
| 10 day old seedlings | 0.42 | 0.48 | 14.28[c] | 0.56 | 0.69 | 23.21[c] | 0.73 | 1.10 | 50.68[c] |
| 15 day old seedlings | 0.72 | 0.77 | 6.94[d] | 0.91 | 1.03 | 13.18[d] | 1.17 | 1.51 | 29.05[d] |
| **CD** | **0.01** | | | **0.008** | | | **0.008** | | |

- DAC – Days after cocultivation
- Values are the mean of fifteen replications

**Table 9. Changes in shoot biomass with *P. indica* colonization**

| Shoot biomass (g/plant) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Treatments** | **7 DAC** | | | **15 DAC** | | | **20 DAC** | | |
| | **Control** | **Treated** | **% increase** | **Control** | **Treated** | **% increase** | **Control** | **Treated** | **% increase** |
| 5 day old seedlings | 0.56 | 0.67 | 19.64[b] | 0.79 | 1.26 | 59.49[a] | 1.13 | 2.02 | 78.76[b] |
| 7 day old seedlings | 0.90 | 1.12 | 24.44[a] | 1.28 | 1.96 | 53.12[b] | 1.45 | 2.62 | 80.68[a] |
| 10 day old seedlings | 1.09 | 1.26 | 15.59[c] | 1.27 | 1.58 | 24.40[c] | 1.64 | 2.78 | 69.51[c] |
| 15 day old seedlings | 1.21 | 1.32 | 9.09[d] | 1.84 | 2.13 | 15.76[d] | 2.04 | 3.16 | 54.90[d] |
| **CD** | **0.008** | | | **0.005** | | | **0.005** | | |

- DAC – Days after cocultivation
- Values are the mean of fifteen replications

**Table 10. Changes in number of secondary roots with *P. indica* colonization**

| Number of secondary roots | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Treatments** | **7 DAC** | | | **15 DAC** | | | **20 DAC** | | |
| | **Control** | **Treated** | **% increase** | **Control** | **Treated** | **% increase** | **Control** | **Treated** | **% increase** |
| 5 day old seedlings | 8.67 | 10.71 | 23.52[a] | 12.34 | 19.56 | 58.53[a] | 15.12 | 26.24 | 73.54[a] |
| 7 day old seedlings | 10.17 | 12.24 | 20.35[b] | 14.16 | 21.20 | 49.68[b] | 17.32 | 29.68 | 71.36[b] |
| 10 day old seedlings | 11.26 | 13.21 | 17.31[c] | 16.32 | 21.86 | 33.94[c] | 17.28 | 27.34 | 58.21[c] |
| 15 day old seedlings | 16.28 | 18.13 | 11.36[d] | 18.42 | 22.66 | 23.01[d] | 21.27 | 28.43 | 33.66[d] |
| **CD** | **0.018** | | | **0.010** | | | **0.015** | | |

- DAC – Days after cocultivation
- Values are the mean of fifteen replications

**Table 11. Changes in number of tertiary roots with *P. indica* colonization**

| Number of tertiary roots | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Treatments | 7 DAC | | | 15 DAC | | | 20 DAC | | |
| | Control | Treated | % increase | Control | Treated | % increase | Control | Treated | % increase |
| 5 day old seedlings | 13.16 | 19.29 | 46.58[a] | 23.21 | 40.47 | 74.36[a] | 28.12 | 53.84 | 91.46[a] |
| 7 day old seedlings | 14.78 | 21.43 | 44.99[b] | 25.19 | 43.46 | 72.52[b] | 36.13 | 68.21 | 88.79[b] |
| 10 day old seedlings | 24.19 | 32.37 | 35.17[c] | 34.35 | 49.80 | 44.97[c] | 42.36 | 73.99 | 74.66[c] |
| 15 day old seedlings | 37.41 | 41.89 | 11.97[d] | 43.19 | 56.46 | 30.72[d] | 49.27 | 83.54 | 69.55[d] |
| **CD** | **0.017** | | | **0.022** | | | **0.028** | | |

- DAC – Days after cocultivation
- Values are the mean of fifteen replications

**Table 12. Changes in number of root hairs per unit area with *P. indica* colonization**

| Root hairs per unit area (number/ $cm^2$) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Treatments** | **7 DAC** | | | **15 DAC** | | | **20 DAC** | | |
| | **Control** | **Treated** | **% increase** | **Control** | **Treated** | **% increase** | **Control** | **Treated** | **% increase** |
| 5 day old seedlings | 5.85 | 7.16 | 22.30[a] | 11.24 | 18.42 | 63.87[a] | 14.35 | 24.42 | 70.17[a] |
| 7 day old seedlings | 5.96 | 7.36 | 21.81[b] | 11.67 | 18.48 | 58.35[b] | 15.14 | 26.56 | 75.42[b] |
| 10 day old seedlings | 7.45 | 8.83 | 18.52[c] | 12.27 | 14.98 | 22.08[c] | 16.14 | 23.82 | 47.58[c] |
| 15 day old seedlings | 11.81 | 12.98 | 9.90[d] | 16.67 | 18.88 | 13.25[d] | 19.21 | 26.17 | 36.23[d] |
| **CD** | **0.021** | | | **0.036** | | | **0.050** | | |

- DAC – Days after cocultivation
- Values are the mean of fifteen replications

**Table 13. Changes in number of leaves with *P. indica* colonization**

| Number of leaves | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Treatments** | **7 DAC** | | | **15 DAC** | | | **20 DAC** | | |
| | **Control** | **Treated** | **% increase** | **Control** | **Treated** | **% increase** | **Control** | **Treated** | **% increase** |
| 5 day old seedlings | 3.33 | 3.9 | 17.11[a] | 7.54 | 9.88 | 31.03[a] | 9.07 | 12.88 | 42.01[a] |
| 7 day old seedlings | 3.81 | 4.43 | 16.27[b] | 7.87 | 10.29 | 30.74[b] | 9.28 | 12.84 | 38.36[b] |
| 10 day old seedlings | 4.61 | 5.24 | 13.66[c] | 8.75 | 10.71 | 22.40[c] | 10.59 | 13.87 | 30.97[c] |
| 15 day old seedlings | 5.65 | 6.02 | 6.54[d] | 9.36 | 10.48 | 11.96[d] | 11.48 | 13.92 | 21.25[d] |
| **CD** | **0.031** | | | **0.030** | | | **0.059** | | |

- DAC – Days after cocultivation
- Values are the mean of fifteen replications

**Table 14. Changes in leaf area with *P. indica* colonization**

| Leaf area ($cm^2$) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Treatments** | **7 DAC** | | | **15 DAC** | | | **20 DAC** | | |
| | **Control** | **Treated** | **% increase** | **Control** | **Treated** | **% increase** | **Control** | **Treated** | **% increase** |
| 5 day old seedlings | 6.25 | 7.65 | 22.4[a] | 11.12 | 16.31 | 46.67[a] | 12.23 | 19.88 | 62.55[a] |
| 7 day old seedlings | 7.46 | 9.08 | 21.71[b] | 12.26 | 17.44 | 42.25[b] | 13.67 | 21.85 | 59.83[b] |
| 10 day old seedlings | 10.29 | 12.11 | 17.68[c] | 13.61 | 17.61 | 23.51[c] | 14.25 | 19.98 | 40.21[c] |
| 15 day old seedlings | 13.32 | 14.47 | 8.63[d] | 15.66 | 18.23 | 16.41[d] | 18.37 | 23.85 | 29.83[d] |
| **CD** | **0.050** | | | **0.043** | | | **0.057** | | |

- DAC – Days after cocultivation
- Values are the mean of fifteen replications

*P. indica* treated cowpea plants showed an increase in leaf area compared to that of control and per cent increase ranged from 8.63 per cent to 62.55 per cent. Highest per cent increase in leaf area was observed for five days old seedlings at twenty days after co-cultivation followed by seven days old seedlings at twenty days after co-cultivation. Lowest value (8.63) was obtained for fifteen days old seedlings at twenty days after co-cultivation (Table 14).

## 4.5. MANAGEMENT OF BICMV USING BOTANICALS AND FUNGAL ROOT ENDOPHYTES

### 4.5.1. Screening potential botanicals against BICMV in *C. amaranticolor*

Screening of AVPs against BICMV infection revealed that treatments with different plant extracts inhibited the virus ranging from 21.7 per cent to 94.84 per cent at three different concentrations in three different methods of application i.e, pre, post and simultaneous inoculation (Table 15). Per cent inhibition was found to be increased with increase in the concentration. Among the seven plant extracts tested, more than 70 per cent inhibition of the virus could be noticed in *P. niruri* and *B. diffusa.*

*P. niruri* extract recorded more than 90 per cent inhibition in pre, post and simultaneous inoculation treatment (Plate 13). In pre inoculation treatment with *P.niruri*, inhibition over control was found to be maximum (94.84 per cent) at 5 per cent concentration followed by 94.08 per cent at 2.5 per cent concentration. In pre inoculation treatment with *P. niruri*, 90.46 per cent inhibition over control was found even at 1% concentration and the per cent inhibition increased with increase in concentration. In simultaneous inoculation the per cent inhibition over control was 90.05 percent at 5 per cent concentration followed by 85.54 per cent and 83.19 per cent at 2.5 and 1 per cent respectively. *P. niruri* extract recorded an inhibition of

**Table 15. Effect of botanicals having antiviral properties on BlCMV in local lesion host, *C. amaranticolor***

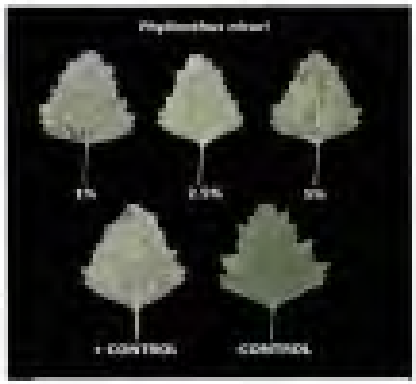
| Botanicals | Method of application | Per cent inhibition over control | | |
|---|---|---|---|---|
| | | 1% | 2.5% | 5% |
| *Boerhaavia diffusa* | Pre inoculation | 83.12(65.80)$^{de}$ | 88.99(70.71)$^{c}$ | 89.67(71.32)$^{c}$ |
| | Simultaneous | 74.84(59.93)$^{hi}$ | 82.45(65.27)$^{ef}$ | 79.40(63.06)$^{g}$ |
| | Post inoculation | 72.03(58.10)$^{ij}$ | 83.91(66.39)$^{de}$ | 88.79(70.50)$^{c}$ |
| *Phyllanthus niruri* | Pre inoculation | 90.46(72.06)$^{bc}$ | 94.08(76.03)$^{a}$ | 94.84(76.97)$^{a}$ |
| | Simultaneous | 83.19(65.84)$^{de}$ | 85.54(67.72)$^{d}$ | 90.05(71.77)$^{c}$ |
| | Post inoculation | 85.32(67.54)$^{d}$ | 89.50(71.14)$^{c}$ | 93.01(74.74)$^{ab}$ |

| | | | | |
|---|---|---|---|---|
| *Calotropis gigantea* | Pre inoculation | 63.92(53.11)$^{op}$ | 80.11(63.58)$^{fg}$ | 75.01(60.03)$^{h}$ |
| | Simultaneous | 30.53(33.56)$^{}$ | 60.34(51.00)$^{qrs}$ | 56.49(48.75)$^{t}$ |
| | Post inoculation | 49.64(44.82)$^{uv}$ | 66.44(54.63)$^{lmno}$ | 65.02(53.77)$^{no}$ |
| *Psidium guajava* | Pre inoculation | 23.52(29.03)$^{}$ | 61.74(51.82)$^{pq}$ | 69.85(56.72)$^{jk}$ |
| | Simultaneous | 21.38(27.55)$^{}$ | 39.65(39.04)$^{y}$ | 46.52(43.03)$^{x}$ |
| | Post inoculation | 30.05(33.25)$^{}$ | 55.24(48.03)$^{t}$ | 50.34(45.21)$^{u}$ |
| *Zingiber officinale* | Pre inoculation | 32.32(34.66)$^{@}$ | 37.48(37.76)$^{yz}$ | 63.94(53.12)$^{p}$ |
| | Simultaneous | 34.32(35.88)$^{@}$ | 65.66(54.15)$^{mno}$ | 48.89(44.38)$^{uvw}$ |
| | Post inoculation | 32.16(34.57)$^{@}$ | 44.07(41.61)$^{x}$ | 61.55(51.71)$^{pq}$ |

| | | | | |
|---|---|---|---|---|
| *Aloe vera* | Pre inoculation | 49.26(44.59)$^{uv}$ $_{w}$ | 72.01(58.10)$^{ij}$ | 80.11(63.58)$^{fg}$ |
| | Simultaneous | 21.36(27.54)$^{\sim}$ | 58.83(50.11)$^{rs}$ | 68.72(56.02)$^{kl}$ |
| | Post inoculation | 32.16(34.57)$^{}$ | 46.87(43.22)$^{vw@}$ | 67.14(55.06)$^{klm}$ |
| *Euphorbia hirta* | Pre inoculation | 61.05(51.41)$^{qr}$ | 68.38(55.81)$^{klm}$ | 73.55(59.09)$^{hi}$ |
| | Simultaneous | 35.86(36.80)$^{z}$ | 39.65(39.04)$^{y}$ | 67.17(55.07)$^{klmno}$ |
| | Post inoculation | 49.66(44.82)$^{uv}$ | 69.91(56.77)$^{jk}$ | 65.05(53.80)$^{n}$ |
| **CD (0.05)** | | **(2.85)** | | |

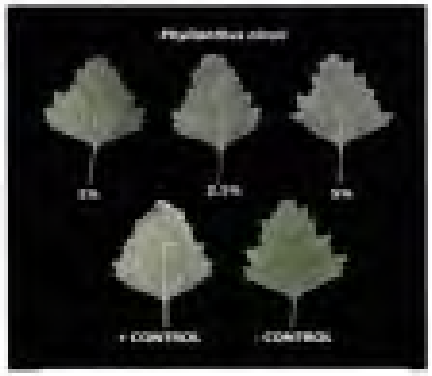
- Values are the mean of three replications and are expressed as per cent inhibition over control.
- Values in parentheses are transformed values

**Plate 13. Effect of *Phyllanthus niruri* extract on BlCMV in local lesion host, *C. amaranticolor***



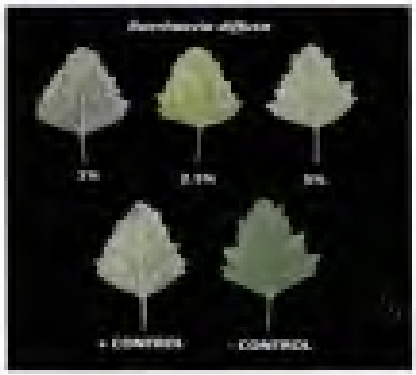
13a. Pre inoculation

13b. Simultaneous inoculation



13c. Post inoculation

**Plate 14. Effect of *Boerhaavia diffusa* extract on BlCMV in local lesion host, *C. amaranticolor***

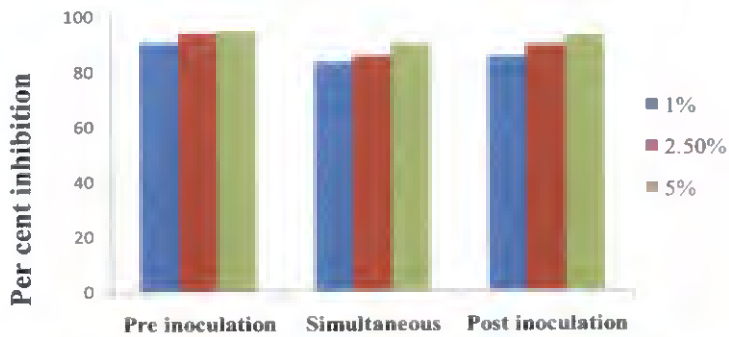


14a. Pre inoculation

14b. Simultaneous inoculation

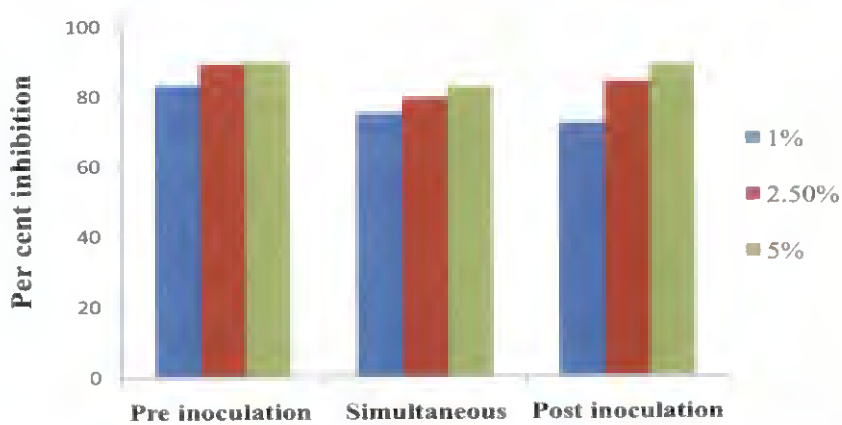
14c. Post inoculation

**Fig. 1. Effect of *Boerhaavia diffusa* extract on *C. amaranticolor***




Method of application

**Fig. 2. Effect of *Phyllanthus niruri* extract on *C. amaranticolor***




Method of application

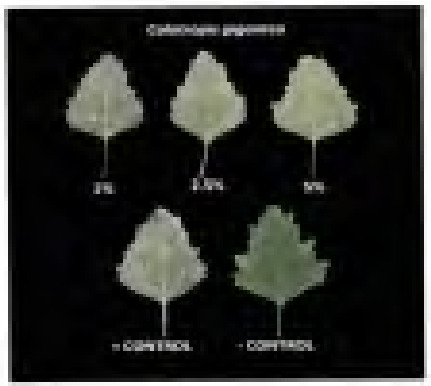
93.01, 89.50 and 85.32 per cent at 5, 2.5 and 1 per cent respectively. There were statistical difference among these concentrations tested for inhibition of the virus.

The next most effective treatment that inhibited the virus was *B. diffusa* (Plate 14). It could inhibit the virus significantly even at 1 per cent concentration at different methods of treatments. At all concentrations, *B. diffusa* inhibited the virus to an extend of more than 70 per cent over control irrespective of the method of applications. In pre inoculation 89.67 per cent inhibition was observed at 5 per cent concentration followed by 88.99 per cent and 83.12 per cent at 2.5 and 1 per cent respectively. In simultaneous inoculation studies maximum inhibition over control (82.45 per cent) was observed at 2.5 per cent followed by 79.40 and 74.84 per cent at 5 and 1 per cent respectively. Maximum inhibition of 88.79 per cent was recorded at 5 per cent concentration followed by 83.91 and 72.03 per cent at 2.5 and 1 per cent respectively.

*Calotropis gigantea* extract exhibited maximum virus inhibition of 80.11 per cent at 2.5 per cent concentration in pre inoculation treatment. In pre inoculation treatment, per cent inhibition over control at 5 and 1 per cent are 75.01 and 63.92 per cent respectively. In simultaneous inoculation 56.49, 60.34 and 30.53 per cent inhibition of virus over control was observed at 5, 2.5 and 1 per cent concentration. In post inoculation treatment, 65.02, 66.44 and 49.64 per cent inhibition of virus was recorded at 5, 2.5 and 1 per cent concentration respectively (Plate 15).

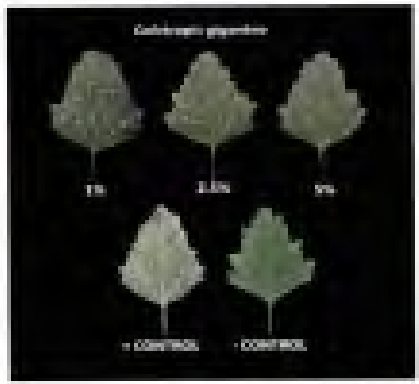
*Psidium guajava* showed significant virus inhibition at higher concentration. In pre inoculation 69.85, 61.74 and 23.52 per cent inhibition of virus was observed at 5, 2.5 and 1 per cent concentration respectively. In simultaneous inoculation 46.2, 39.65 and 21.38 per cent inhibition of virus was recorded at 5, 2.5 and 1 per cent concentration respectively. An inhibition of 50.34, 55.24 and 30.05 per cent was observed at 5, 2.5 and 1 per cent concentration respectively in post inoculation studies (Plate 16).

**Plate 15. Effect of *Calotropis gigantea* extract on BlCMV in local lesion host, *C. amaranticolor***



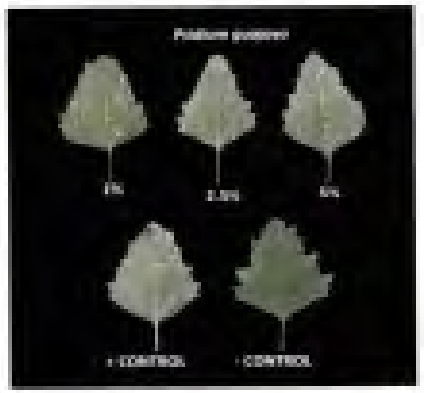
**15a. Pre inoculation**

**15b. Simultaneous inoculation**

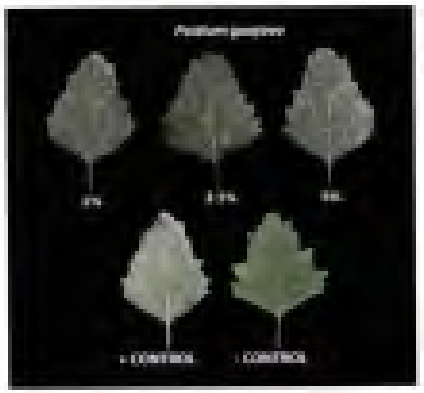


**15c. Post inoculation**

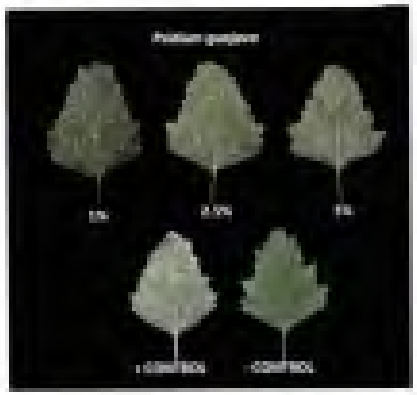
**Plate 16. Effect of *Psidium guajava* extract on BlCMV in local lesion host, *C. amaranticolor***



**16a. Pre inoculation**



**16b. Simultaneous inoculation**



**16c. Post inoculation**

**Fig. 3 Effect of *Calotropis gigantea* extract on *C. amaranticolor***

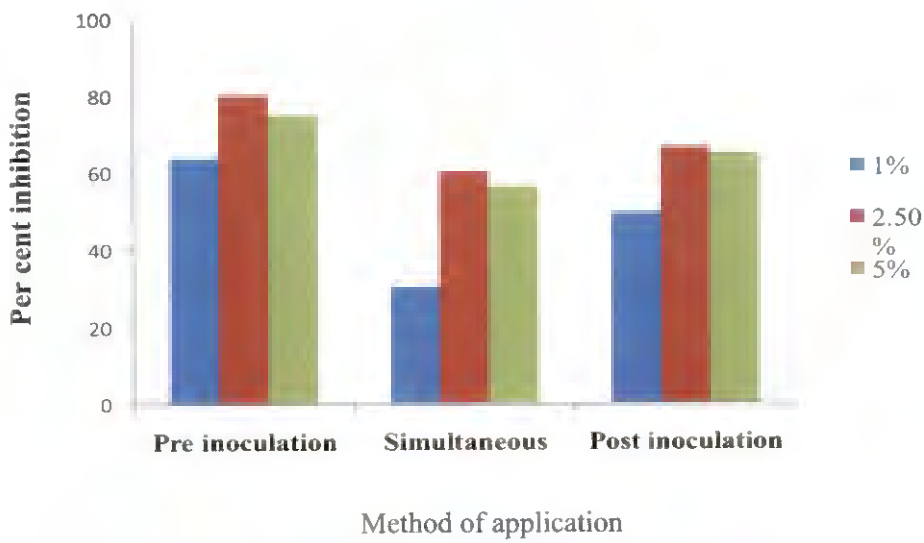
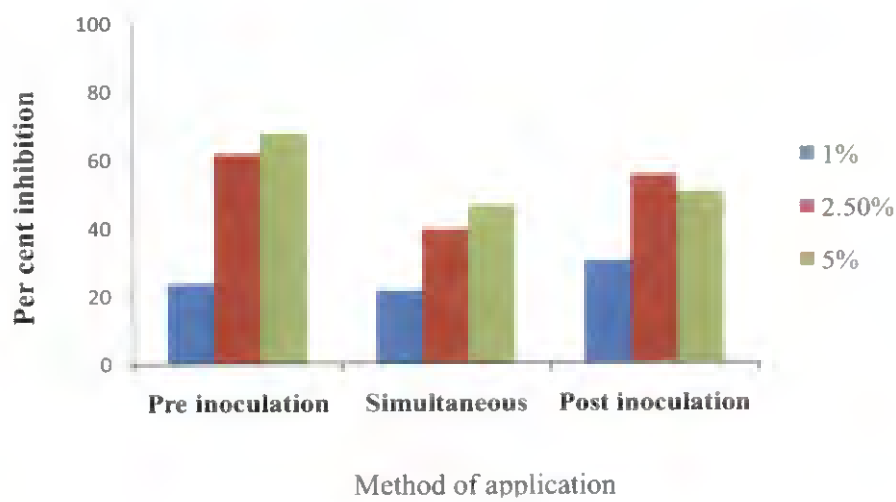



**Fig. 4 Effect of *Psidium guajava* extract on *C. amaranticolor***



In pre inoculation *Zingiber officinale* extract exhibited an inhibition of 63.94, 37.48 and 32.32 per cent at 5, 2.5 and 1 per cent respectively. In simultaneous inoculation, maximum inhibition of virus (65.66 per cent) was observed at 2.5 per cent followed by 48.89 and 34.32 per cent at 5 and 1 per cent concentration respectively. In post inoculation, 61.55, 44.07 and 32.16 per cent inhibition was recorded at 5, 2.5 and 1 per cent respectively (Plate 17).

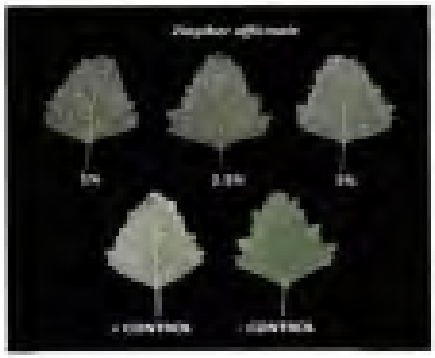
*Aloe vera* extracts recorded a maximum inhibition of virus (80.11 per cent) at 5 per cent concentration followed by 72.01 per cent at 2.5 per cent and 49.26 per cent at 1 per cent concentration in pre inoculation treatment. In simultaneous inoculation, 68.72, 58.83 and 21.36 per cent inhibition over control was observed at 5, 2.5 and 1 per cent concentration respectively. An inhibition of 67.14, 46.87 and 32.16 per cent was recorded at 5, 2.5 and 1 per cent respectively in post inoculation studies (Plate 18).

*Euphorbia hirta* was also found to be inhibiting the virus significantly above 35 per cent in different methods of application at all different concentrations. In pre inoculation, 73.55, 68.38 and 61.05 per cent inhibition of virus was observed at 5, 2.5 and 1 per cent respectively. Per cent inhibition over control at 5, 2.5 and 1 per cent concentration was recorded as 67.17, 39.65 and 35.86 respectively in simultaneous inoculation and 65.05, 69.91 and 49.66 respectively in post inoculation (Plate 19).

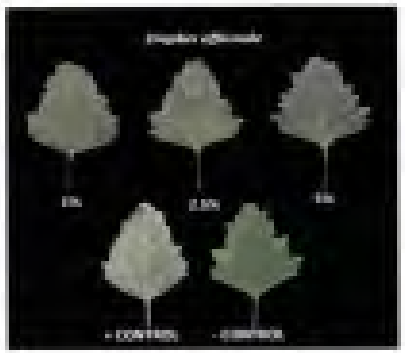
*Calotropis gigantea, Zingiber officinale* and *Euphorbia hirta* were also found to be inhibiting the virus significantly above 30 per cent in different methods of application at all different concentrations. *Calotropis gigantea* showed maximum virus inhibition of 80.12 per cent at 2.5 per cent of pre inoculation. *Psidium guajava* and *Aloe vera* inhibited the virus above 20 per cent with a maximum virus inhibition at 5 per cent concentration.

In pre inoculation treatment maximum inhibition was observed by *P. niruri* followed by *Boerhaavia diffusa, Calotropis gigantea* and *Euphorbia hirta* at all

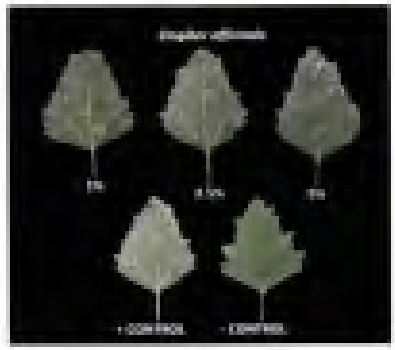
Plate 17. Effect of *Zingiber officinale* extract on BlCMV in local lesion host, *C. amaranticolor*



17a. Pre inoculation

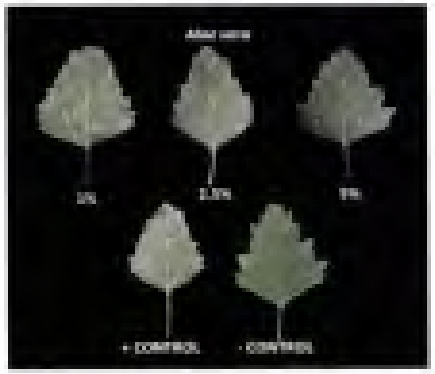


17b. Simultaneous inoculation



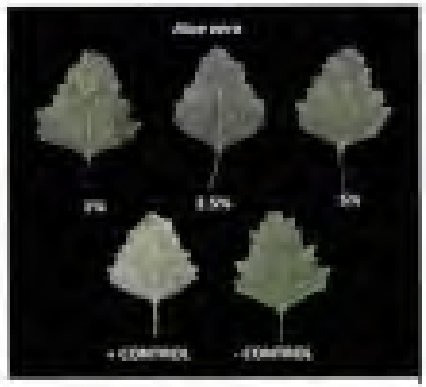
17c. Post inoculation

Plate 18. Effect of *Aloe vera* extract on BlCMV in local lesion host, *C. amaranticolor*



18a. Pre inoculation

18b. Simultaneous inoculation

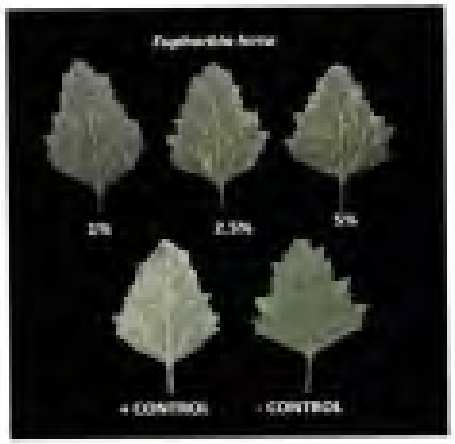


18c. Post inoculation

**Plate 19. Effect of *Euphorbia hirta* etract on BlCMV in local lesion host, *C. amaranticolor***


19a. Pre inoculation


19b. Simultaneous inoculation




19c. Post inoculation

**Fig. 5. Effect of *Zingiber officinale* extract on *C. amaranticolor***

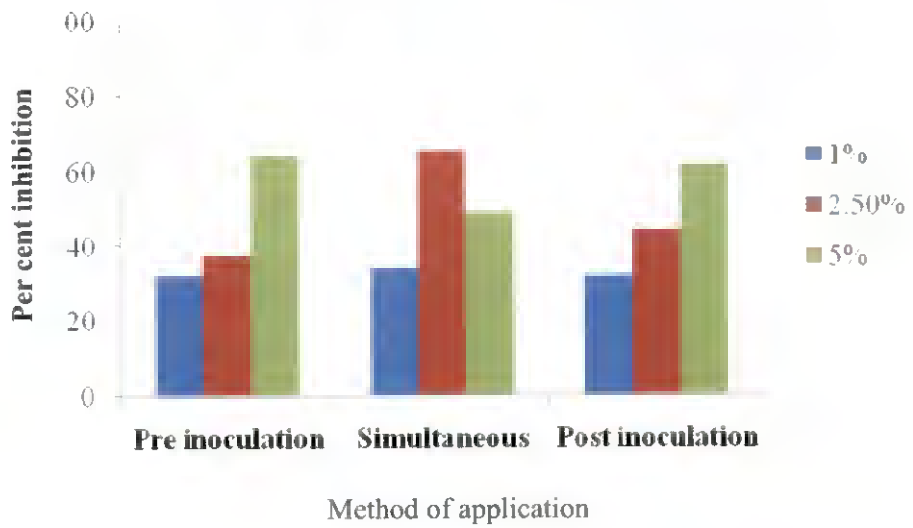



**Fig. 6. Effect of *Aloe vera* extract on *C. amaranticolor***

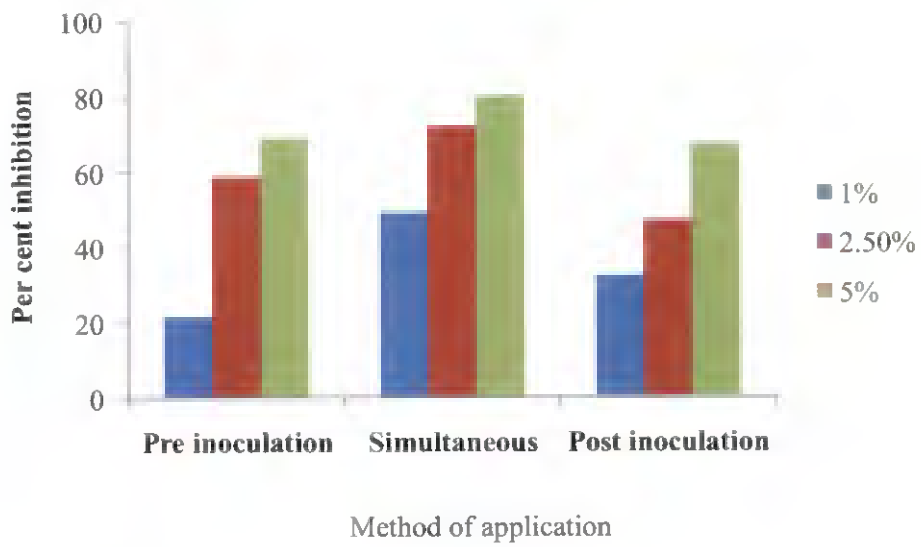
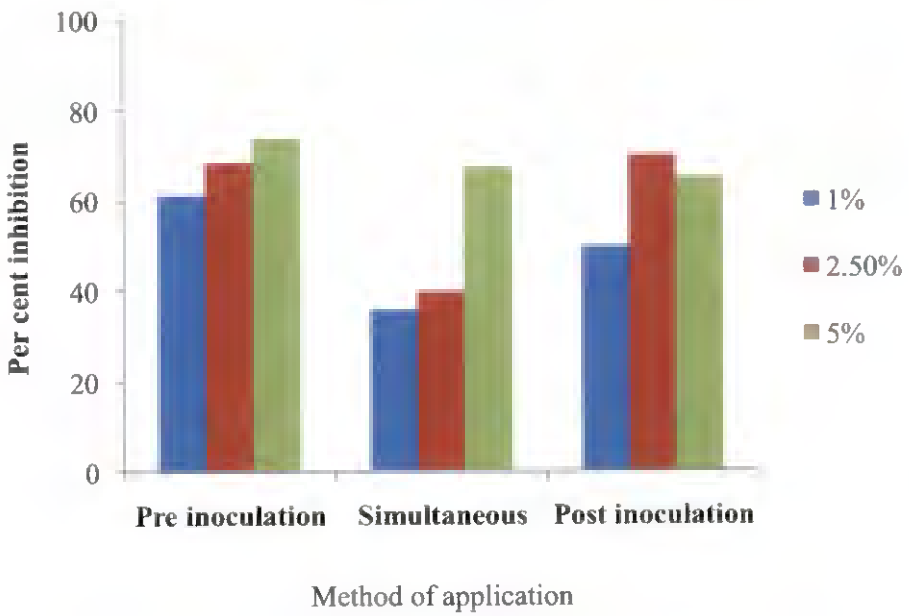


**Fig. 7. Effect of *Euphorbia hirta* extract on *C. amaranticolor***



concentrations. Maximum inhibition at 5 per cent concentration was observed to be on par with *P. niruri* at 2.5 per cent concentration in pre-inoculation treatment. *Zingiber officinale, Psidium guajava* and *Aloe vera* showed significant virus inhibition at higher concentration.

Simultaneous inoculation treatment of virus and plant extracts revealed that *P. niruri* was the best one to inhibit the virus at all concentrations followed by *Calotropis gigantea, Zingiber officinale* and *Aloe vera.* In *P. niruri* about 90 percent inhibition was recorded at 5 per cent and more than 80 per cent inhibition was observed at 1 and 2.5 per cent concentration.

Data on post-inoculation treatment with plant extracts revealed that *P. niruri* extract was the best one to inhibit the virus at all concentrations. This was followed by *B. diffusa, Euphorbia hirta* and *Calotropis gigantea. P. niruri* recorded about 85 per cent of inhibition on virus even at 1 per cent concentration. The per cent inhibition of the virus increased with increase in concentrations of plant extracts.

### 4.5.2. Evaluation of AVP and fungal endophyte treated Cowpea Plants

The botanical and fungal root endophyte that showed best results in local lesion host were selected to find out its efficiency in cowpea. Vulnerability index was calculated and it was found that AVP treated plants showed less vulnerable to infection even at 30 DAI. This was followed by *P. indica* treated plants. In both the cases, treatments that were applied before challenge inoculation of the virus showed less symptom expression than the post inoculation studies (Table 16). Compared to control both the treatments showed less vulnerability index at all stages of observation. In all the treatments, plants were found prone to infection with increase in plant age.

**Table 16. Vulnerability index of AVP and fungal endophyte treated cowpea plants**

| Treatments | Days after inoculation | | | |
|---|---|---|---|---|
| | 5 | 10 | 15 | 30 |
| Healthy | 0 | 0 | 0 | 0 |
| Control (*P. niruri* pre inoculation) | $21.40^{a}$ | $35.23^{c}$ | $45.33^{d}$ | $63.78^{a}$ |
| *P. niruri* (pre inoculation) | $7.14^{g}$ | $14.28^{h}$ | $20.00^{h}$ | $27.52^{g}$ |
| Control (*P. indica* pre inoculation) | $20.20^{c}$ | $35.24^{b}$ | $45.82^{a}$ | $63.68^{b}$ |
| *P. indica*(pre inoculation) | $9.52^{f}$ | $15.23^{g}$ | $21.66^{g}$ | $34.13^{e}$ |
| Control (*P. niruri* post inoculation) | $21.38^{b}$ | $35.28^{a}$ | $45.36^{c}$ | $63.78^{a}$ |
| *P. niruri* (post inoculation) | $11.90^{e}$ | $17.67^{f}$ | $23.33^{f}$ | $33.26^{f}$ |
| Control (*P. indica* post inoculation) | $20.23^{c}$ | $35.21^{d}$ | $45.78^{b}$ | $63.76^{c}$ |
| *P. indica*(post inoculation) | $16.66^{d}$ | $24.50^{e}$ | $33.62^{e}$ | $47.43^{d}$ |
| **CD(0.05)** | **0.037** | **0.003** | **0.009** | **0.009** |

- Values are the mean of three replications

## 4.6. Biochemical Changes of Host Pathogen Interaction

### 4.6.1. Estimation of total soluble protein

Estimation of protein content was carried out as per the procedure given by Bradford (1976) and results are summarized in Table 17 and fig. 8.

The result indicated that there was a progressive increase in the total protein content in treated, virus inoculated and healthy plants with increase in the age of plants. The virus inoculated cowpea plants showed higher values of protein content when compared to uninoculated healthy plants throughout the period of observation.

Among the treatments, cowpea plants pre inoculated with *Phyllanthus niruri* recorded highest values for protein content at all intervals of observation with an increasing trend from 5 DAI (1.78 mg $g^{-1}$) to 30 DAI (4.60 mg $g^{-1}$). This was followed by cowpea plants pre treated with *Piriformospora indica.* Similar trend was observed in treatment with *Piriformospora indica* also. Treatment with *P. indica* also showed similar trend from 5 DAI (1.64 mg $g^{-1)}$ to 30 DAI (3.91 mg $g^{-1}$).

In post inoculation, protein content of cowpea plants pre treated with *P. niruri* increased from 5 DAI (1.58 mg $g^{-1}$) and attained a peak at 30 DAI (3.12 mg $g^{-1}$). Total protein content of cowpea plants pre inoculated with *P. indica*, increased from 5 DAI(1.28 mg $g^{-1}$) to reach the maximum at 30 DAI (2.78 mg $g^{-1}$).

### 4.6.2 Estimation of Total Sugars

The estimation of total sugars was done as per the procedure given by (Hedge and Hofreiter, 1962) and the results are presented in the Table 18 and Fig. 9.

The trait showed an increasing trend up to 15 DAI and thereafter a reduction was observed at 30 DAI. Highest values of sugar content was observed for healthy plant samples compared to the virus inoculated and treated cowpea plants.

**Table 17. Changes in total soluble protein content of cowpea against BlCMV**

| Treatments | Total soluble protein content (mg $g^{-1}$ fresh weight of tissue) | | | |
|---|---|---|---|---|
| | **5 DAI** | **10 DAI** | **15 DAI** | **30 DAI** |
| Healthy | 1.10[f] | 1.45[h] | 1.83[i] | 2.26[g] |
| Control (*P. niruri* pre inoculation) | 1.38[d] | 1.98[d] | 2.77[d] | 3.72[d] |
| *P. niruri* (pre inoculation) | 1.78[a] | 2.82[a] | 3.93[a] | 4.60[a] |
| Control (*P. indica* pre inoculation) | 1.36[d] | 1.96[e] | 2.66[g] | 3.84[c] |
| *P. indica*(pre inoculation) | 1.64[b] | 2.64[b] | 3.61[b] | 3.91[b] |
| Control (*P. niruri* post inoculation) | 1.36[d] | 1.99[d] | 2.75[e] | 3.75[d] |
| *P. niruri* (post inoculation) | 1.58[c] | 2.13[c] | 2.97[c] | 3.12[e] |
| Control (*P. indica* post inoculation) | 1.37[d] | 1.95[f] | 2.68[f] | 3.81[c] |
| *P. indica*(post inoculation) | 1.28[e] | 1.46[g] | 1.93[h] | 2.78[f] |
| **CD(0.05)** | **0.033** | **0.009** | **0.009** | **0.033** |

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

**Table 18. Changes in total sugar content of cowpea against BlCMV**

| Treatments | Total sugar content (mg $g^{-1}$ fresh weight of tissue) | | | |
|---|---|---|---|---|
| | 5 DAI | 10 DAI | 15 DAI | 30 DAI |
| Healthy | $24.60^{a}$ | $28.30^{a}$ | $31.40^{b}$ | $36.40^{a}$ |
| Control (*P. niruri* pre inoculation) | $18.00^{g}$ | $20.10^{h}$ | $26.70^{f}$ | $25.20^{e}$ |
| *P. niruri* (pre inoculation) | $23.00^{b}$ | $27.80^{b}$ | $33.20^{a}$ | $29.42^{b}$ |
| Control ( *P. indica* pre inoculation) | $16.17^{h}$ | $20.73^{f}$ | $23.82^{h}$ | $20.34^{h}$ |
| *P. indica*(pre inoculation) | $19.30^{d}$ | $23.20^{d}$ | $27.70^{d}$ | $26.20^{d}$ |
| Control ( *P. niruri* post inoculation) | $18.22^{f}$ | $20.30^{f}$ | $26.91^{e}$ | $24.42^{f}$ |
| *P. niruri* (post inoculation) | $20.60^{c}$ | $23.90^{g}$ | $28.60^{c}$ | $27.37^{c}$ |
| Control ( *P. indica* post inoculation) | $16.21^{h}$ | $20.71^{c}$ | $23.85^{h}$ | $20.74^{h}$ |
| *P. indica*(post inoculation) | $18.90^{e}$ | $21.70^{e}$ | $24.60^{g}$ | $21.37^{g}$ |
| **CD(0.05)** | **0.071** | **0.072** | **0.075** | **0.075** |

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

**Fig. 8 Changes in total soluble protein content of cowpea leaves against BlCMV**

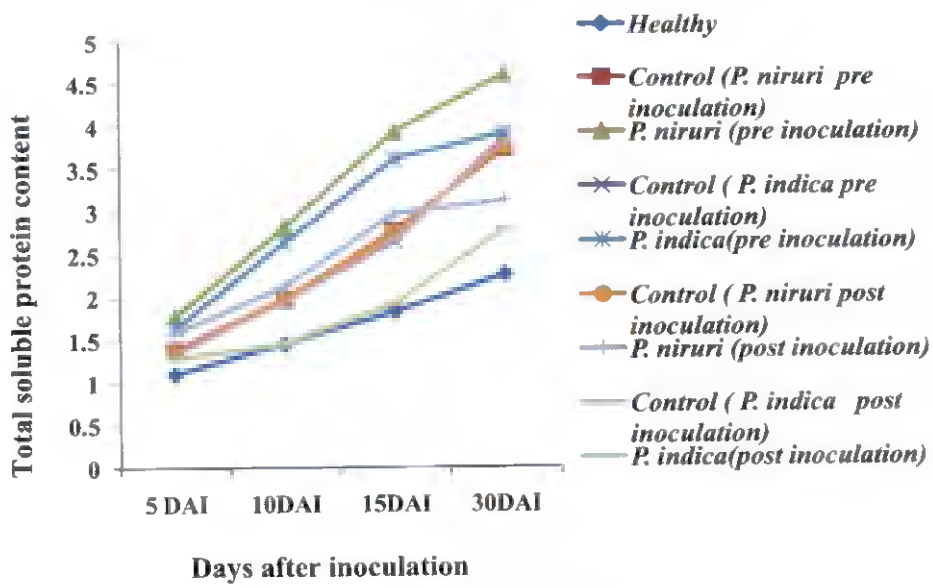
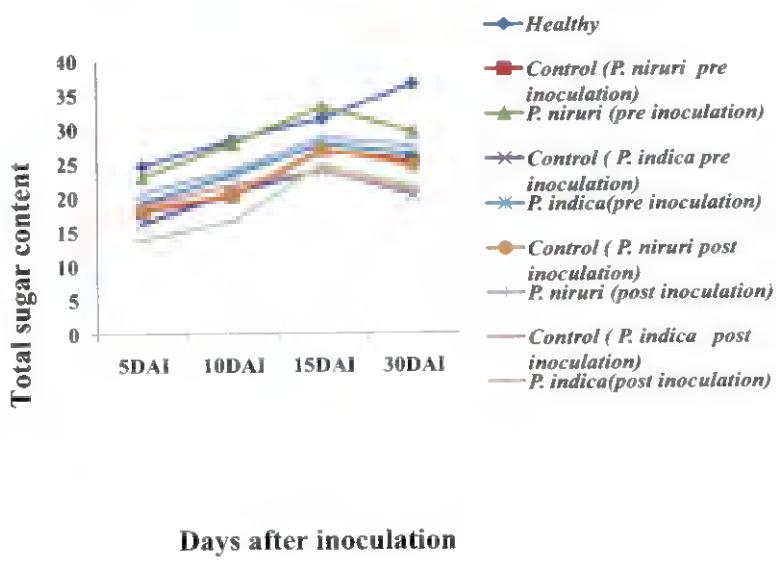



**Fig. 9 Changes in total sugar content of cowpea leaves against BlCMV**



In pre inoculation treatments, total sugar content of cowpea plants pre treated with *P. niruri* was found to increase from 5 DAI (23.0 mg $g^{-1}$) and attained a peak at 15 DAI (33.2 mg $g^{-1}$) and there after declined to 29.42 mg $g^{-1}$ at 30 DAI. In case of treatments with *P. indica,* a similar trend in total sugar was observed with a peak value at 15 DAI (27.70 mg $g^{-1}$).

In Post-inoculation, *P. niruri* treated cowpea plants showed similar trend as that of pre inoculation treatments, with a maximum sugar content of 28.6 mg $g^{-1}$ at 15 DAI and treatments with *P. indica* recorded a maximum sugar content of 24.6 mg $g^{-1}$ at 15 DAI.

**4.6.3. Estimation of Reducing Sugar**

Reducing sugar content was estimated by dinitrosalicylic acid method and results are summarized in Table 19 and Fig. 10.

The studies on reducing sugar content in cowpea revealed that there was an increase in reducing sugar up to 10 DAI for all the treatments and thereafter it was found to decline at 15 DAI to 30DAI. Healthy cowpea plants showed higher reducing sugar content than cowpea plants inoculated with virus alone in both method of applications.

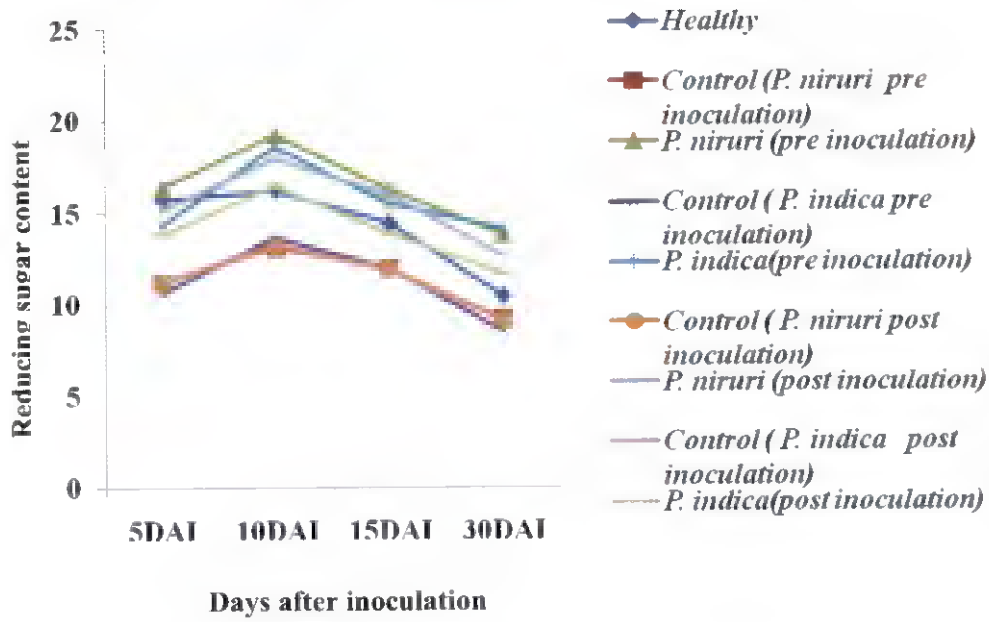
Among the two method of application, pre inoculation treatment recorded high sugar content than post inoculation. In pre inoculation treatment, cowpea plants treated with *P. niruri* recorded highest reducing sugar content up to 15 DAI. At 30 DAI, the highest reducing sugar content was recorded by cowpea plants pre treated with *P. indica.* In *P. niruri* treated cowpea plants, from 5 DAI to 10 DAI an increase in sugar content was observed with a peak value (19.25 mg $g^{-1}$) at 10 DAI thereafter gradual decrease in reducing sugar content was observed on 15 DAI (16.29 mg $g^{-1}$) and 30 DAI (13.83 mg $g^{-1}$). *P. indica* treated cowpea plants also exhibited a similar trend with its highest value at 10 DAI (18.56 mg $g^{-1}$).

**Table 19. Changes in reducing sugar content of cowpea against BICMV**

| Treatments | Reducing sugar content (mg $g^{-1}$ fresh weight of tissue) | | | |
|---|---|---|---|---|
| | 5 DAI | 10 DAI | 15 DAI | 30 DAI |
| Healthy | 15.77[b] | 16.23[e] | 14.46[d] | 10.45[e] |
| Control (*P. niruri* pre inoculation) | 11.22[g] | 13.13[i] | 11.98[f] | 9.17[f] |
| *P. niruri* (pre inoculation) | 16.38[a] | 19.25[a] | 16.29[a] | 13.83[b] |
| Control (*P. indica* pre inoculation) | 10.56[i] | 13.67[f] | 11.95[g] | 8.50[i] |
| *P. indica*(pre inoculation) | 14.34[d] | 18.56[b] | 15.44[c] | 14.12[a] |
| Control (*P. niruri* post inoculation) | 11.19[h] | 13.16[h] | 11.95[g] | 9.12[h] |
| *P. niruri* (post inoculation) | 15.18[c] | 17.96[c] | 16.02[b] | 12.67[c] |
| Control (*P. indica* post inoculation) | 11.23[f] | 13.31[g] | 11.85[h] | 9.14[g] |
| *P. indica*(post inoculation) | 13.78[e] | 16.45[d] | 13.87[e] | 11.67[d] |
| **CD(0.05)** | **0.303** | **0.48** | **0.18** | **0.18** |

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

**Fig. 10 Changes in reducing sugar content of cowpea leaves against BICMV**



Post inoculation treatment showed lesser reducing sugar content than pre inoculation. In post inoculation, cowpea plants treated with *P. niruri* and *P. indica* recorded maximum value 17.96 mg $g^{-1}$ and 16.45 mg $g^{-1}$ respectively at 10 DAI and later reduced at 15 DAI to 30DAI.

**4.6.4. Estimation of Phenol**

Estimation of phenol was carried out as per the procedure given by Bray and Thorpe (1954) and results are summarized in Table 20 and Fig. 11.

Application of AVP'S on cowpea cv. Sharika inoculated with BlCMV induced higher accumulation of phenolics. The maximum accumulation was observed at 15 DAI and declined thereafter. Uninoculated healthy plants recorded lower phenol content when compared to virus inoculated cowpea plants.

In pre inoculation, *P. niruri* treated cowpea plants recorded an increase in phenol content from 5 DAI to 15 DAI. At 15 DAI maximum total phenol content recorded was 1.53 mg $g^{-1}$ and in case of *P. indica* treated plants, gradual increase in phenol content was observed from 5 DAI to 30 DAI. In *P. indica* inoculated cowpea plants, maximum phenol content (1.58 mg g-1) was recorded at 30 DAI.

Post inoculation treatments recorded a low phenol content in all the treatments when compared to pre inoculation. In *P. niruri* treated plants, increase in phenol content was observed with increase in the age of the plant, upto 15 DAI and there after reduced. But in the case of *P. indica* inoculated plants a continuous increase of phenol content was observed from 5 DAI to 30 DAI. AVP and endophyte treated plants showed higher accumulation of phenol when compared to virus inoculated and healthy plant samples.

**Table 20. Changes in Phenol content of cowpea against BlCMV**

| Treatments | Phenol content (mg $g^{-1}$ fresh weight of tissue) | | | |
|---|---|---|---|---|
| | 5 DAI | 10 DAI | 15 DAI | 30 DAI |
| Healthy | $0.16^{i}$ | $0.59^{h}$ | $0.66^{f}$ | $0.32^{h}$ |
| Control (*P. niruri* pre inoculation) | $0.31^{h}$ | $0.94^{g}$ | $1.10^{e}$ | $0.68^{e}$ |
| *P. niruri* (pre inoculation) | $0.86^{a}$ | $1.41^{a}$ | $1.53^{a}$ | $1.19^{c}$ |
| Control (*P. indica* pre inoculation) | $0.36^{f}$ | $0.96^{e}$ | $1.16^{de}$ | $0.64^{g}$ |
| *P. indica*(pre inoculation) | $0.77^{b}$ | $1.23^{b}$ | $1.4^{b}$ | $1.58^{a}$ |
| Control (*P. niruri* post inoculation) | $0.35^{g}$ | $0.99^{d}$ | $1.14^{de}$ | $0.67^{e}$ |
| *P. niruri* (post inoculation) | $0.58^{d}$ | $1.14^{c}$ | $1.26^{c}$ | $0.83^{d}$ |
| Control (*P. indica* post inoculation) | $0.37^{e}$ | $0.95^{f}$ | $1.18^{d}$ | $0.65^{f}$ |
| *P. indica*(post inoculation) | $0.61^{c}$ | $0.99^{d}$ | $1.17^{de}$ | $1.29^{b}$ |
| **CD(0.05)** | **0.009** | **0.009** | **0.004** | **0.007** |

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

**Fig. 11 Changes in phenol content of cowpea leaves against BICMV**

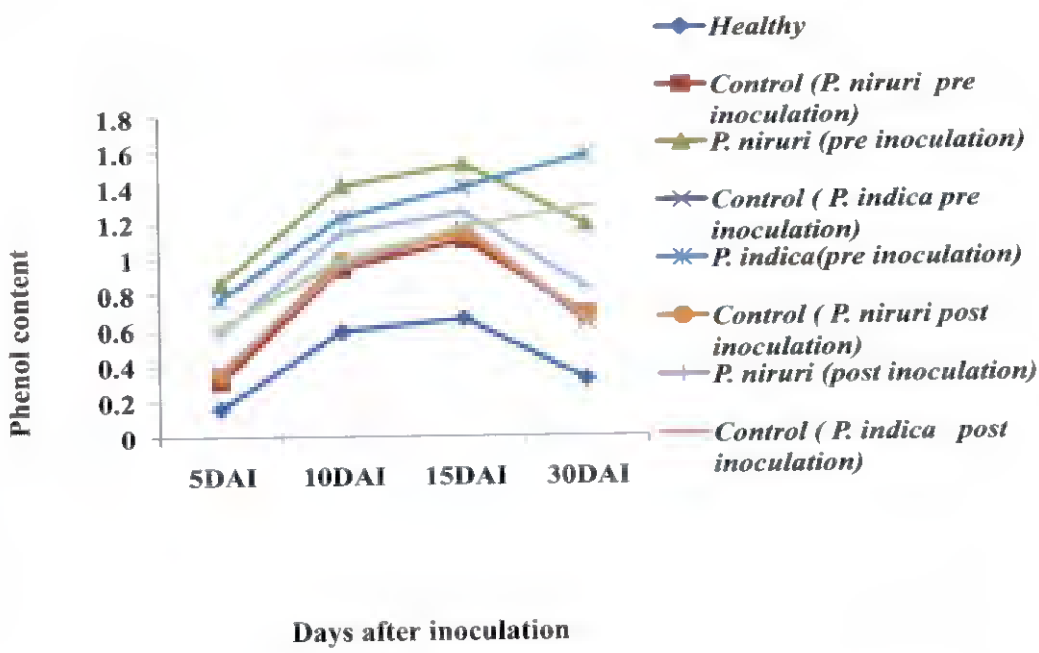
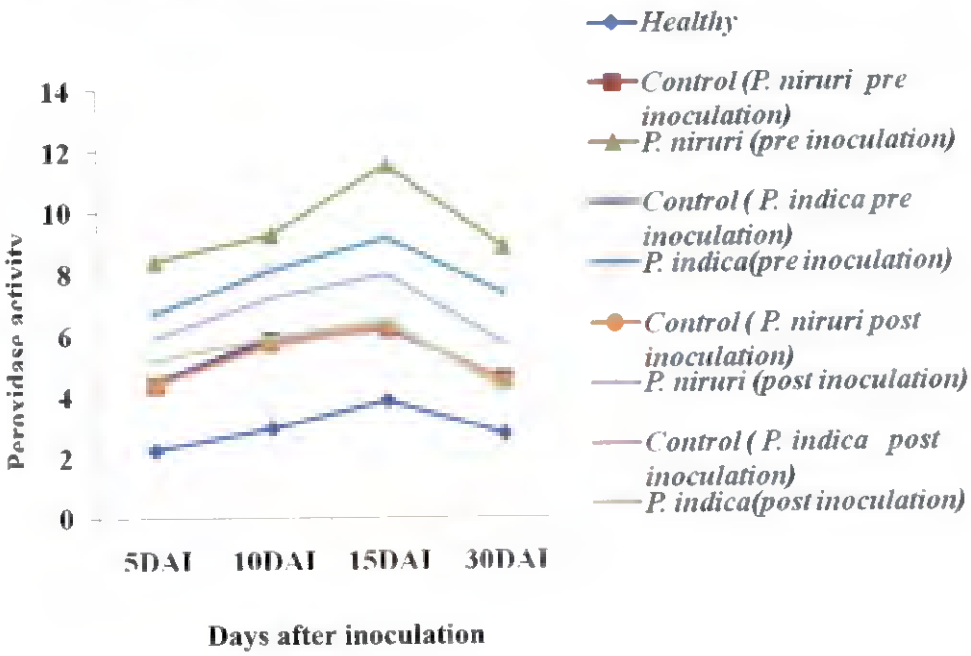



**Fig. 12 Changes in Peroxidase activity of cowpea leaves against BICMV**



**4.6.5. Estimation of Peroxidase (PO)**

The changes in enzyme activity on the inoculated and uninoculated plants of both genotypes are presented in the Table 21 and Fig. 12.

AVP treatment against BlCMV revealed that they induce defence mechanisms in plants challenged with BlCMV. Though peroxidase activity was observed in healthy plants, significantly higher activity was observed in the case of *P. niruri* and *P. indica* treated cowpea plants. Peroxidase activity recorded a progressive increase up to 15 DAI and therafter it declined. In both pre and post inoculation treatments BlCMV inoculated cowpea plants recorded higher PO activity compared to virus inoculated and healthy plants.

In pre inoculation, *P. niruri* treated plants induced highest PO activity compared to *P. indica* inoculated cowpea plants. PO activity of *P. niruri* treated plants increased from 5 DAI (8.4 $min^{-1}$ $g^{-1}$) to a peak value at 15 DAI (11.49 $min^{-1}$ $g^{-1}$) and later declined to 8.83 $min^{-1}$ $g^{-1}$ at 30 DAI. *P. indica* inoculated plants also exhibited a similar trend with an increase in PO activity from 5 DAI (6.7 $min^{-1}$ $g^{-1}$) to a maximum value at 15 DAI (9.13 $min^{-1}$ $g^{-1}$) which declined slightly thereafter.

In post inoculation, treated plants, virus inoculated control plants and healthy plants showed a steady increase of PO activity from 5 DAI to 15 DAI and a decline thereafter. Cowpea plants treated with *P. niruri* recorded an increase in PO activity from 5 DAI (5.9 $min^{-1}$ $g^{-1}$) to 15 DAI (7.92 $min^{-1}$ $g^{-1}$) followed by a decline at 30 DAI (5.71 $min^{-1}$ $g^{-1}$). *P. indica* treated plants also significantly increased the PO activity until 15 DAI but less than the *P. niruri* treated plants and showed a declining trend after 15 DAI.

**Table 21. Changes in Peroxidase activity of cowpea against BlCMV**

| Treatments | Peroxidase activity ($min^{-1} g^{-1}$ fresh weight of tissue) | | | |
|---|---|---|---|---|
| | 5 DAI | 10 DAI | 15 DAI | 30 DAI |
| Healthy | 2.20[g] | 2.90[g] | 3.80[g] | 2.70[h] |
| Control (*P. niruri* pre inoculation) | 4.35[f] | 5.79[e] | 6.17[f] | 4.50[d] |
| *P. niruri* (pre inoculation) | 8.40[a] | 9.25[a] | 11.49[a] | 8.83[a] |
| Control (*P. indica* pre inoculation) | 4.46[e] | 5.87[d] | 6.15[f] | 4.51[d] |
| *P. indica*(pre inoculation) | 6.70[b] | 8.10[b] | 9.13[b] | 7.32[b] |
| Control (*P. niruri* post inoculation) | 4.39[f] | 5.66[f] | 6.25[e] | 4.42[f] |
| *P. niruri* (post inoculation) | 5.90[c] | 7.20[c] | 7.92[c] | 5.71[c] |
| Control (*P. indica* post inoculation) | 4.33[f] | 5.71[f] | 6.28[c] | 4.46[e] |
| *P. indica*(post inoculation) | 5.17[d] | 5.89[d] | 6.44[d] | 4.16[g] |
| **CD(0.05)** | **0.064** | **0.057** | **0.033** | **0.033** |

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

**4.6.6. Estimation of Polyphenol Oxidase (PPO)**

Estimation of polyphenol oxidase activity was conducted as per the procedure given by Mayer *et al.* (1965) and results are given in Table 22 and Fig. 13.

Results indicate that there was an increasing trend in the PPO activity of AVP and endophyte treated cowpea plants challenged with BlCMV, BlCMV inoculated control plants and uninoculated healthy plants until 15 DAI. As a result of inoculation with BlCMV, PPO activity was continuously increased until 15 DAI, which was more than the uninoculated healthy plants. But the rate of increase showed a declining trend after 15 DAI.

In pre inoculation application of AVP treated plants inoculated with BlCMV, PPO activity significantly increased over the control plants. PPO activity of *P. niruri* treated plants recorded an increase from 5 DAI (2.1 $min^{-1}$ $g^{-1}$) to 15 DAI (3.9 $min^{-1}$ $g^{-1}$) and thereafter declined to 1.3 $min^{-1}$ $g^{-1}$ at 30 DAI. Similar trend was observed in the case of *P. indica* inoculated cowpea plants with an increase from 5 DAI (1.6 $min^{-1}$ $g^{-1}$) to 15 DAI (3.13 $min^{-1}$ $g^{-1}$) and further reduced to 2.97 $min^{-1}$ $g^{-1}$ at 30 DAI.

Post inoculation treatments of AVP's showed an increase in PPO activity compared to virus inoculated and healthy plants but less than the pre inoculation treatments. Cowpea plants post inoculated with *P. niruri* recorded an increase in PPO activity from 5 DAI (1.18 $min^{-1}$ $g^{-1}$) to 15 DAI (2.92 $min^{-1}$ $g^{-1}$) and later declined to 0.73 $min^{-1}$ $g^{-1}$ at 30 DAI. Similarly *P. indica* treated plants exhibited an increase in PPO activity from 5 DAI (0.93 $min^{-1}$ $g^{-1}$) to a peak value at 15 DAI (2.57 $min^{-1}$ $g^{-1}$) and reduced to 2.11 $min^{-1}$ $g^{-1}$ at 30 DAI.

**Table 22. Changes in Polyphenol oxidase activity of cowpea against BlCMV**

| Treatments | Polyphenol oxidase activity ($min^{-1} g^{-1}$ fresh weight of tissue) | | | |
|---|---|---|---|---|
| | **5 DAI** | **10 DAI** | **15 DAI** | **30 DAI** |
| Healthy | $0.10^{g}$ | $0.70^{f}$ | $1.30^{g}$ | $0.45^{f}$ |
| Control (*P. niruri* pre inoculation) | $0.60^{e}$ | $0.90^{e}$ | $1.75^{f}$ | $0.50^{e}$ |
| *P. niruri* (pre inoculation) | $2.10^{a}$ | $3.30^{a}$ | $3.90^{a}$ | $1.30^{c}$ |
| Control (*P. indica* pre inoculation) | $0.56^{f}$ | $0.87^{e}$ | $1.81^{e}$ | $0.51^{e}$ |
| *P. indica*(pre inoculation) | $1.60^{b}$ | $2.26^{b}$ | $3.13^{b}$ | $2.97^{a}$ |
| Control (*P. niruri* post inoculation) | $0.59^{ef}$ | $0.86^{e}$ | $1.83^{e}$ | $0.54^{e}$ |
| *P. niruri* (post inoculation) | $1.18^{c}$ | $1.96^{c}$ | $2.92^{c}$ | $0.73^{d}$ |
| Control (*P. indica* post inoculation) | $0.63^{e}$ | $0.88^{e}$ | $1.85^{e}$ | $0.54^{e}$ |
| *P. indica*(post inoculation) | $0.93^{d}$ | $1.52^{d}$ | $2.57^{d}$ | $2.11^{b}$ |
| **CD(0.05)** | **0.057** | **0.057** | **0.044** | **0.044** |

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

**Fig. 13 Changes in Polyphenol oxidase activity of cowpea leaves against BlCMV**

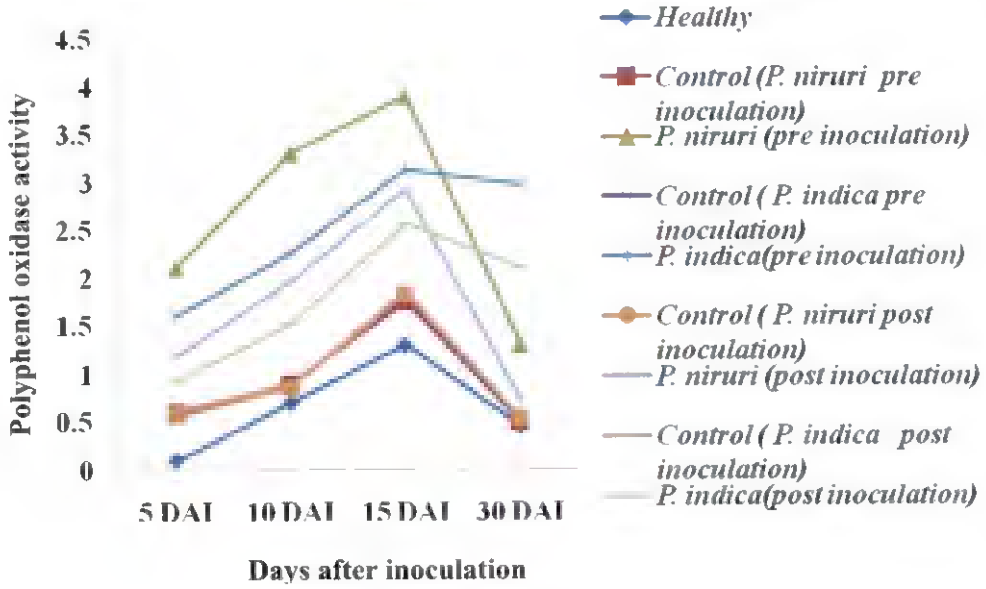
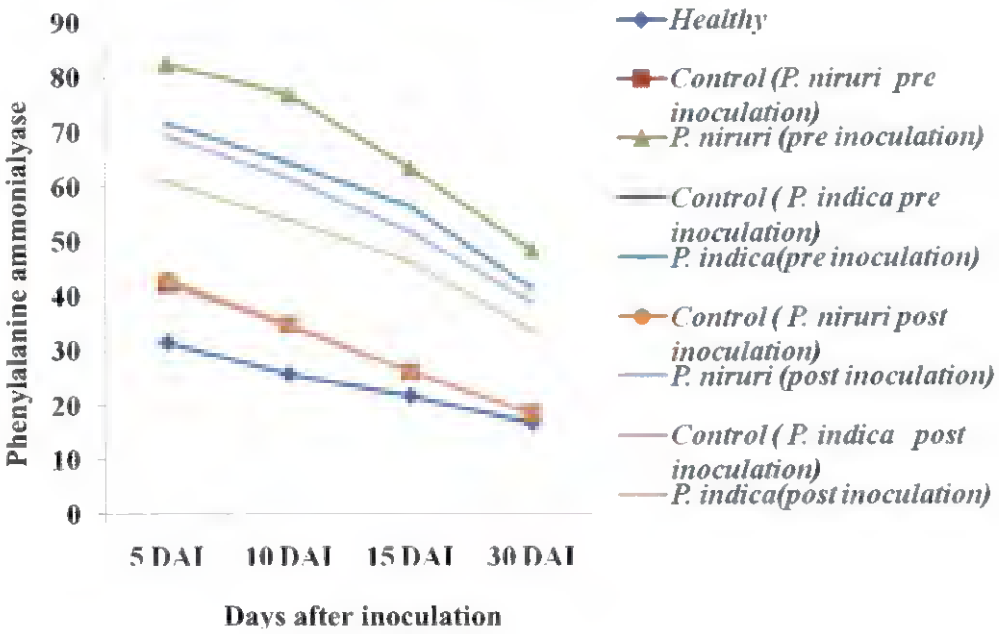



**Fig. 14 Changes in Phenylalanine ammonialyase activity of cowpea leaves against BlCMV**



### 4.6.7. Estimation of Phenylalanine Ammonialyase (PAL)

Phenylalanine ammonialyase activity was estimated as per the procedure developed by Dickerson *et al.* (1984) and results are presented in Table 23 and Fig. 14.

Studies on the effect of antiviral principles and fungal endophyte on cowpea challenge inoculated with BlCMV revealed an increased accumulation of PAL than virus inoculated and healthy cowpea plants. AVP and endophyte treated plants, inoculated and healthy plants all exhibited a decreasing trend in PAL accumulation from 5 DAI to 30 DAI. Healthy plants recorded the lowest PAL accumulation compared to BlCMV inoculated cowpea plants.

In pre inoculation, *P. niruri* treated cowpea plants challenged with BlCMV recorded the maximum PAL activity of 82.5 $\mu g\ g^{-1}\ min^{-1}$ on 5 DAI followed by a decline throughout the period of observation. *P. indica* treated plants recorded highest accumulation of PAL at 5 DAI (71.66 $\mu g\ g^{-1}\ min^{-1}$).

In post inoculation, the enzyme activity of *P. niruri* treated plants recorded peak values at 5 DAI (69.16 $\mu g\ g^{-1}\ min^{-1}$) followed by a reduction in the activity until 30 DAI. Similar to this *P. indica* inoculated plants exhibited highest PAL activity at 5 DAI (60.83 $\mu g\ g^{-1}\ min^{-1}$). PAL activity was found to be higher in pre inoculated plants than post inoculated plant samples.

### 4.6.8. Electrophoretic Analysis of Proteins by SDS-PAGE

Electrophoretic analysis of proteins were conducted on cowpea against BlCMV treated with *P. niruri* and *P. indica* along with virus inoculated and healthy cowpea plants of Sharika at different time intervals *viz.*, five day, ten day, fifteen day and thirty days after inoculation. Many proteins were induced in response to various treatments.

**Table 23. Changes in Phenylalanine ammonialyase activity of cowpea against BICMV**

| Treatments | Phenylalanine ammonialyase activity (µg $g^{-1}$ $min^{-1}$ fresh weight of tissue) | | | |
|---|---|---|---|---|
| | **5 DAI** | **10 DAI** | **15 DAI** | **30 DAI** |
| Healthy | 31.66[i] | 25.83[i] | 21.76[i] | 16.66[i] |
| Control (*P. niruri* pre inoculation) | 42.5[h] | 34.72[h] | 26.12[h] | 18.33[g] |
| *P. niruri* (pre inoculation) | 82.5[a] | 76.82[a] | 63.25[a] | 48.33[a] |
| Control (*P. indica* pre inoculation) | 42.56[g] | 34.82[g] | 26.18[e] | 18.31[h] |
| *P. indica*(pre inoculation) | 71.66[b] | 64.27[b] | 56.18[b] | 41.32[b] |
| Control (*P. niruri* post inoculation) | 43.11[f] | 34.86[f] | 26.13[g] | 18.44[e] |
| *P. niruri* (post inoculation) | 69.16[c] | 61.31[c] | 51.62[c] | 38.71[c] |
| Control (*P. indica* post inoculation) | 42.63[e] | 34.88[e] | 26.15[f] | 18.35[f] |
| *P. indica*(post inoculation) | 60.83[d] | 53.82[d] | 46.27[d] | 33.82[d] |
| **CD(0.05)** | **0.044** | **0.007** | **0.008** | **0.008** |

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

At 5 DAI, proteins were induced at 45 KDa for all the treatments except for healthy and proteins of 43 KDa were induced in cowpea plants pre and post inoculated with *P. niruri* and *P. indica*. In virus inoculated plants alone, a protein of 29 KDa were induced. In *P. niruri* pre inoculation treatment, extra proteins were observed at 21 KDa and 31 KDa (Plate 20).

Treated cowpea plants at 10 DAI induced proteins of 27 KDa and 30 KDa for all the treatments except healthy. An induction of 97.4 KDa proteins was observed in cowpea plants pre treated with *P. niruri* and in cowpea plants pre and post inoculated with *P. indica*. In all treatments except healthy and pre inoculation treatment of *P. niruri* an extra band of 21 KDa was observed and in pre inoculation treatment of *P. niruri* novel protein bands of 20.5 KDa and 22KDa were observed. In pre and post inoculation treatments of *P. indica* three new proteins were induced between 14.3 KDa and 20.1 KDa (Plate 21).

At 15 DAI, new proteins were induced at 78 KDa, 86 KDa and 97.4 KDa for pre and post inoculation treatments with *P. niruri* and *P. indica* along with the pre and post inoculation control with *P. indica*. In *P. niruri* treated plants, 54 KDa proteins were induced in pre and post inoculation of both control and treated plants. Induced proteins with 20.1 KDa, 22KDa, 25KDa, 27KDa, 29 KDa, 35 KDa, 37KDa and 52 KDa were observed in cowpea plants pre treated with *P. niruri*. In cowpea plants post inoculated with *P. niruri* and pre inoculated with *P. indica*, induction of proteins with 20.1 KDa, 22KDa, 25KDa, 29 KDa and 52 KDa were observed and in cowpea plants post inoculated with *P. indica,* 20.1 KDa and 52 KDa proteins were induced at fifteen days after inoculation (Plate 22).

A decrease in number of protein bands was observed at 30 DAI. In all treatments except healthy, induced proteins of 29 KDa and 35 KDa were observed. In cowpea plants pre treated with *P. niruri*, induction of proteins with 26 KDa, 27 KDa and 66 KDa were observed and in cowpea plants post inoculated with

**Plate 20. Appearance of PR proteins in cowpea leaves inoculated with BlCMV and treated with AVP and Endophyte at 5 DAI**

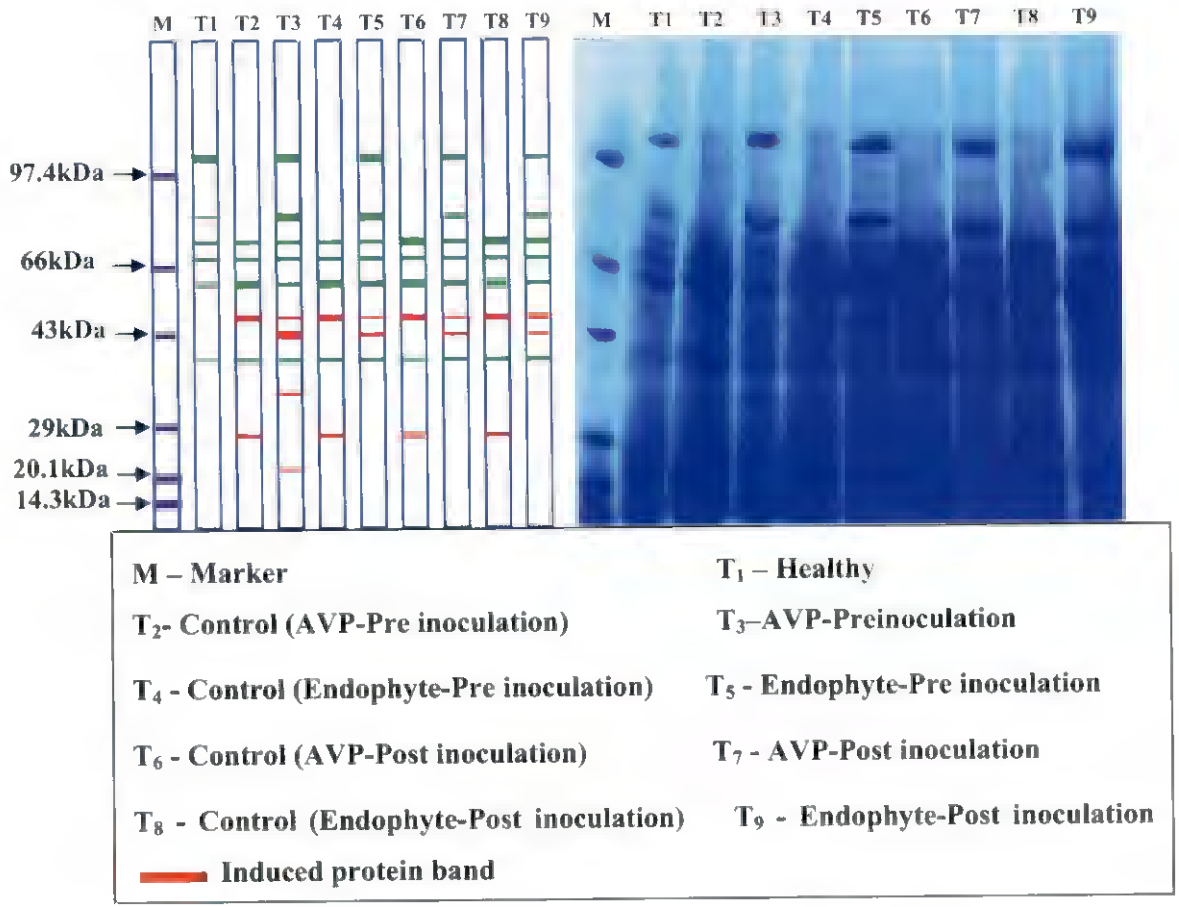
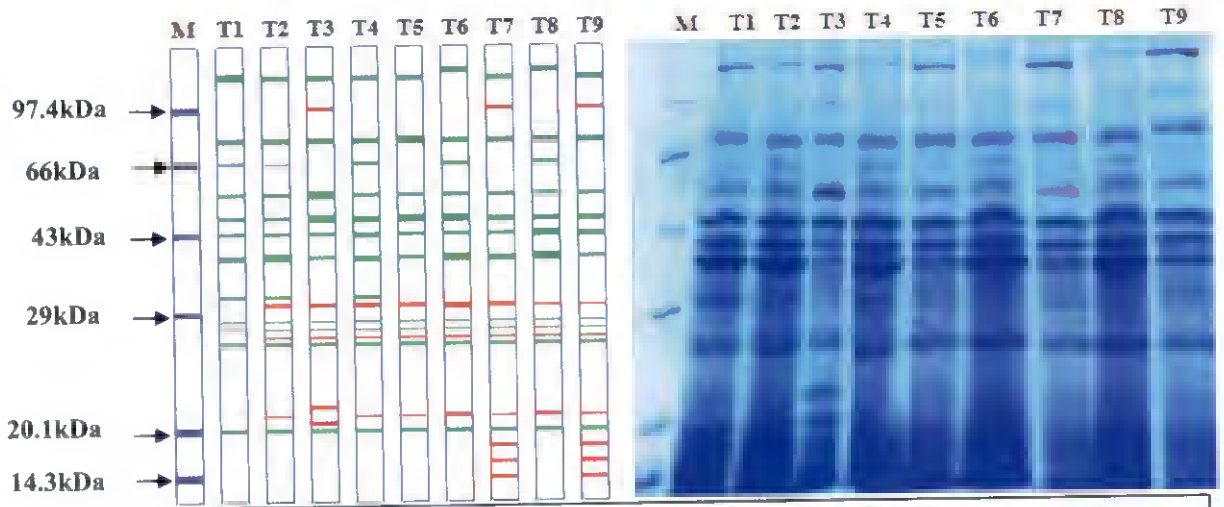


**Plate 21. Appearance of PR proteins in cowpea leaves inoculated with BICMV and treated with AVP and Endophyte at 10 DAI**




M – Marker

$T_1$ – Healthy

$T_2$- Control (AVP-Pre inoculation)

$T_3$–AVP-Preinoculation

$T_4$ - Control (Endophyte-Pre inoculation)

$T_5$ - Endophyte-Pre inoculation

$T_6$ - Control (AVP-Post inoculation)

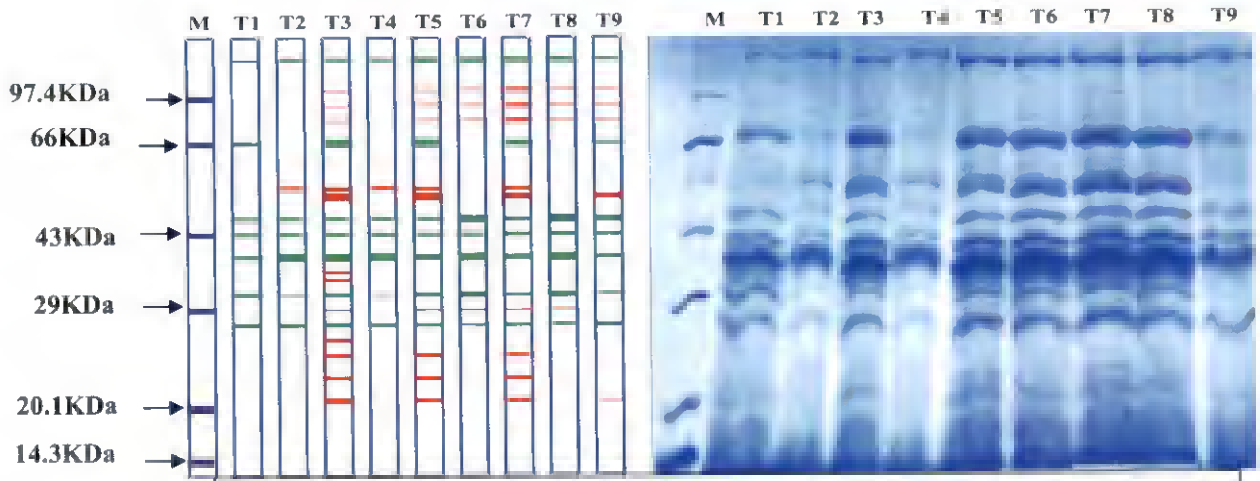
$T_7$ - AVP-Post inoculation

$T_8$ - Control (Endophyte-Post inoculation)

$T_9$ - Endophyte-Post inoculation

Induced protein band

**Plate 22. Appearance of PR proteins in cowpea leaves inoculated with BlCMV and treated with AVP and Endophyte at 15 DAI**




M – Marker

$T_1$ – Healthy

$T_2$- Control (AVP-Pre inoculation)

$T_3$–AVP-Preinoculation

$T_4$ - Control (Endophyte-Pre inoculation)

$T_5$ - Endophyte-Pre inoculation

$T_6$ - Control (AVP-Post inoculation)

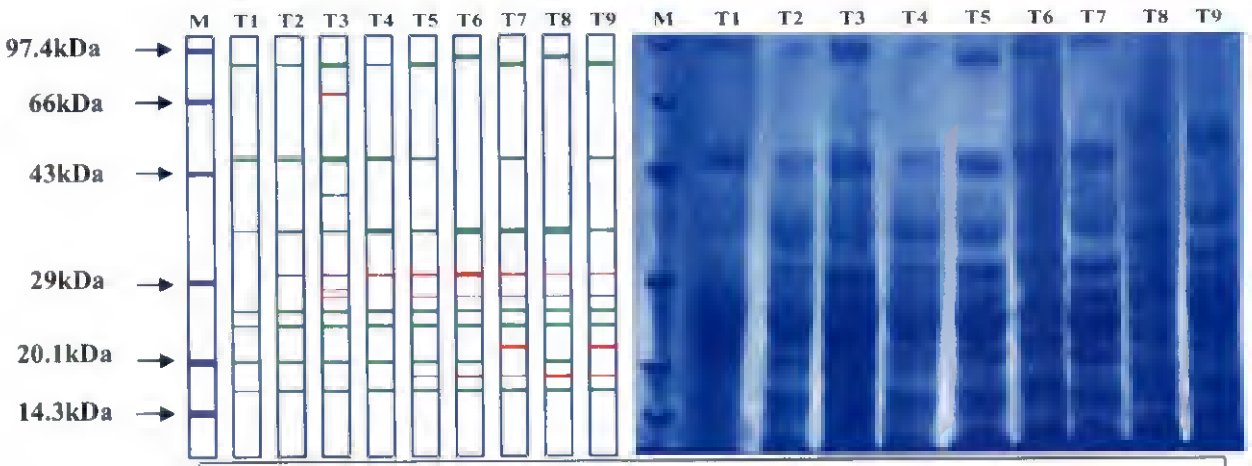
$T_7$ - AVP-Post inoculation

$T_8$ - Control (Endophyte-Post inoculation)

$T_9$ - Endophyte-Post inoculation

Induced protein band

**Plate 23. Appearance of PR proteins in cowpea leaves inoculated with BICMV and treated with AVP and Endophyte at 30 DAI**




M – Marker

$T_1$ – Healthy

$T_2$- Control (AVP-Pre inoculation)

$T_3$–AVP-Preinoculation

$T_4$ - Control (Endophyte-Pre inoculation)

$T_5$ - Endophyte-Pre inoculation

$T_6$ - Control (AVP-Post inoculation)

$T_7$ - AVP-Post inoculation

$T_8$ - Control (Endophyte-Post inoculation)

$T_9$ - Endophyte-Post inoculation

Induced protein band

*P. niruri*,18 KDa and 26 KDa proteins were induced. Cowpea plants pre and post inoculated with *P. indica* exhibited new protein bands with 18 KDa, 26 KDa and 29 KDa molecular weight (Plate 23).

# *Discussion*

# 5. DISCUSSION

The results of the study on the symptomatology, immunological and molecular detection and management of *Cowpea mosaic virus* infecting cowpea are discussed below,

Cowpea (*Vigna unguiculata* (L.) Walp. ) is a grain crop with high protein, minerals and vitamins and is utilized as a fresh vegetable (pods and leaves) and as fodder. Vegetable cowpea can be cultivated throughout the year under Kerala conditions. Diseases and pests are the major constraints of cowpea production. Among the pathogens, viruses are a serious threat to the cultivars. Diseases caused by viruses are the most destructive and are often difficult to control. Detection and management of viral disease has always been a problem to cultivators and research workers.

Taking into consideration the destructive losses caused by the *Cowpea mosaic virus*, symtomatology, immunomolecular detection and management were conducted.

## 5.1. MAINTENANCE OF VIRUS AND ENDOPHYTES

### 5.1.1. Maintenance of Virus

*Cowpea mosaic virus* was maintained in *Chenopodium amaranticolor* and cowpea by repeated transfers through mechanical inoculation. Radhika (1999) reported the maintenance of *Blackeye cowpea mosaic virus* in cowpea and *C. amaranticolor* by sap transmission using 0.01M phosphate buffer. Krishnapriya (2015) reported that the BlCMV was maintained in a susceptible variety, Vellayani Jyothika on sap transmission using 0.01M phosphate buffer.

BlCMV was maintained in cowpea seedlings by mechanical inoculation with BlCMV infected trifoliate leaves of the highly susceptible "Knuckle Purpose Hull"

(Ouattara and Chambliss, 1991). The stock culture of the BlCMV isolate were maintained by frequent mechanical inoculation on healthy susceptible cowpea plants in an insect-free greenhouse (Bashir *et al.*, 2002) .

**5.1.2. Symptomatology**

The results revealed that the cowpea cultivar, Sharika developed initial symptoms of vein clearing after 8 days of inoculation with BlCMV. BlCMV resulted in a wide range of symptoms including initial vein clearing, mosaic, vein banding, reduction in leaf size and distortion. The plants also showed severe stunting and resulted in reduction in number of flowers and pods. Similar observations of vein clearing, mosaic, vein banding and leaf distortion on trifoliate leaves were described by Hao *et al.* (2003). Udayashankar *et al.* (2009) reported mosaic, vein banding, vein clearing, and mottling on the primary leaf followed by mosaic, vein banding, leaf rolling, downward folding of leaves and leaf distortion on the trifoliate leaves due to BlCMV infection.

Radhika (1999) reported dark vein banding, severe mosaic, blistering and distortion of leaves and stunting of the plant as the symptoms. Dark green and light green mottling, mosaic and vein banding on leaves, reduction in leaf size and distortion, accompanied with severe stunting of plants with a reduction in number of flowers and pods were observed in cowpea inoculated with BlCMV (Krishnapriya, 2015).

Contradictory to the present observations, Thottappilly and Rossel (1985) have reported localized symptoms as large reddish, often ring-like lesions which typically spread along the veins, forming a reddish net pattern and systemic symptoms as mottling and green vein banding along with interveinal chlorosis, stunting and leaf distortion due to BlCMV infection in cowpea plants.

Mechanical inoculation of BlCMV in *Chenopodium amaranticolor* leaves resulted in the development of yellow chlorotic lesions after five days of inoculation. BlCMV was biologically purified through a single local lesion technique on *Chenopodium amaranticolor* by mechanical inoculation (Zettler *et al.*, 1967). Radhika (1999) reported the presence of chlorotic local lesions in *C. amaranticolor* inoculated with BlCMV. The leaves of *C. amaranticolor* were inoculated with the BlCMV infected leaf samples from each urd bean samples and the inoculated leaves were observed for local lesions (Udayashankar *et al.*, 2012).

**5.1.3. Maintenance of Fungal Endophytes**

Fungal root endophytes *Piriformospora indica* and *Fusarium pallidoroseum* were maintained in Potato dextrose agar medium by continuous subculturing. Varma *et al.* (1999) reported that the fungus, *P. indica* can be cultivated on complex or minimal substrates. *P. indica* can be easily cultivated on a variety of synthetic media (Oelmuller *et al.*, 2009). Varkey (2016) maintained *P. indica* by continuous subculturing in Potato dextrose agar medium. Vyshakhi (2016) reported that the fungal endophyte, *P. indica* was isolated from plant roots on potato dextrose agar medium and the fungus was maintained on the media by continuous subculturing.

Gokulapalan *et al.* (2006) reported the isolation and maintenance of *F. pallidoroseum* from symptomatic cowpea samples on Potato dextrose agar medium using standard procedures. Gondim *et al.* (2008) isolated *F. pallidoroseum* from fruit rot infected melon fruits on potato dextrose agar medium and the fungus was maintained on the same medium by subculturing procedures. The pathogenic *F. pallidoroseum* from infected chilli fruits showing typical fruit rot symptoms was isolated and maintained on PDA medium (Parey *et al.*, 2013).

## 5.2. DETECTION OF *Cowpea mosaic virus* USING SEROLOGICAL METHOD

### 5.2.1. Enzyme Linked Immunosorbent Assay (ELISA)

DAC-ELISA using polyclonal (H2) and monoclonal (15E6 and 16G5) antibodies specific to BlCMV gave a positive reaction in samples collected from symptomatic plants. Also CABMV specific polyclonal antibody (H4) showed positive reaction to the symptomatic plants however, a negative reaction was observed with monoclonal antibodies (7A10 and 5H5) specific to CABMV, indicating the virus causing mosaic disease in cowpea as BlCMV. In the present study there was cross reaction with PAb specific to CABMV and BlCMV, but the two viruses could be differentiated using MAbs specific to the two viruses respectively. In tune with the results of present study, Hao *et al.* (2003) reported a positive reaction of symptomatic plants with PAbs of BlCMV (H2), CABMV (H4) and MAbs of BlCMV (15E6 and 16G5) whereas negative reaction to the symptomatic plants were observed with MAbs of CABMV (5H5 and 7D5).

Radhika (1999) detected BlCMV in cowpea plants using monoclonal antibodies specific to BlCMV. Puttaraju *et al.* (2004) used antibodies specific to BlCMV for the detection of BlCMV in symptomatic cowpea leaf samples. Among the 136 seedlots of cowpea subjected to the growing-on test, seedlings from 43 cowpea seed lots gave positive reactions in ELISA to BCMV-BlCMV (Udayashankar *et al.*, 2009). Krishnapriya (2015) reported a positive reaction of mosaic symptom in cowpea with polyclonal (H2) and monoclonal (15E6 and 16G5) antibodies of BlCMV, confirming the virus causing mosaic disease of cowpea in Kerala as BlCMV.

### 5.2.2. Dot Immunobinding Assay (DIBA)

DIBA have been used for the identification of *Potyviruses* in cowpea plants (Sidaros *et al.*, 2006; Akinjogunla *et al.*, 2008). In the present study, DIBA was conducted to detect the presence of the virus causing mosaic disease in cowpea, by using the polyclonal antiserum of BlCMV (H2) and CABMV (H4). Both H2 and H4 gave positive colour reaction and a higher mean value of intensity compared to healthy with highest value for H2 (2,866.0) than H4 (1,624.6). The present study revealed that the two PAbs used cross reacted. The highest mean value of intensity for H2 indicated the presence of BlCMV as the mosaic causing virus in cowpea. In line with the present findings, Hao *et al.* (2003) reported a positive reaction to the polyclonal antibodies of both BlCMV (H2) and CABMV (H4).

According to Huguenot *et al.* (1993) *Blackeye cowpea mosaic virus* and *Cowpea aphid borne mosaic virus* are serologically distinguishable with monoclonal antibodies, but may cross-react with polyclonal antibodies. Krishnapriya (2015) reported a positive reaction of the mosaic sample towards the polyclonal antiserum of BlCMV whereas a negative reaction towards the polyclonal antiserum of CABMV, thus confirming the virus causing mosaic of cowpea in Kerala as BlCMV.

## 5.3. DEVELOPMENT OF MOLECULAR PROBE(S) FOR THE DETECTION AND DIAGNOSIS OF *Cowpea mosaic virus*

Detection of mosaic causing virus in cowpea was performed using RT-PCR. The coat protein (CP) gene of BlCMV and CABMV specific primers designed and that obtained from published papers were used for the detection of mosaic causing virus in cowpea. The CP gene specific primer designed resulted in amplified product with a size of ~ 425 bp whereas CP gene specific primer obtained from published paper (Krishnapriya, 2015) resulted in amplified product with a size of ~ 110 bp. RT-PCR results thus confirmed that the mosaic disease of cowpea in Kerala was caused by BlCMV. Krishnapriya (2015) amplified the BlCMV Kerala isolate using

CP specific primer with an amplicon of size 110 bp. This result is in conformity with the results obtained for the BlCMV isolate in the present study.

Udayashankar *et al.* (2009) conducted RT-PCR using both CABMV and BCMV-BlCMV specific primers and no amplification was observed for CABMV primers whereas an amplification of ~ 700bp for BCMV-BlCMV indicated the presence of *Blackeye cowpea mosaic virus* in the sample. Bhadramurthy and Bhat (2009) performed RT-PCR for the detection of BCMV-BlCMV infecting vanilla in Kerala using CP gene specific primer which resulted in amplification of ~ 850bp. Koohapitagtam and Nualsri (2013) performed PCR for the detection of BlCMV in cowpea and yard-long bean. They used CACP1 forward primer and CACP2 reverse primer which resulted in an amplified product of 864 bp.

## 5.4. STANDARDIZATION OF CO-CULTIVATION METHOD

### 5.4.1. Co-cultivation of Plants and Fungal Endophyte in Plant Nutrient Media (PNM)

The present study revealed that the co-cultivation of two leaf stage cowpea with four weeks old *P. indica* and *F. pallidoroseum* conducted in jam bottles showed decay of cowpea plants, which might be due to the insufficiency of space for the trailing type Sharika variety.

Co-cultivation of *Chenopodium amaranticolor* with *P. indica* and *F. pallidoroseum* were also conducted in jam bottles. No fungal colonization was observed within the roots after co-cultivation. Kumar *et al.* (2009) reported family Chenopodiaceae as a non host of the fungal endophyte arbuscular mycorrhizal fungi (AMF) since *Chenopodium amaranticolor* belongs to the family *Chenopodiaceae*, it can be a non host of the fungal endophyte, AMF. According to Qiang *et al.* (2011) *P. indica* is a fungus which resembles AMF. So *C. amaranticolor* might be a non host to *P. indica* also.

### 5.4.2. Co-cultivation of Plants and Fungal Endophytes in Vermiculite - Perlite medium

No root colonization was observed for *Chenopodium amaranticolor* with any of the two fungal endophytes ie, *Fusarium pallidoroseum* and *Piriformospora indica*. According to Kumar *et al.* (2009) *C. amaranticolor* can be a non host of the fungal endophyte, AMF. Since *P. indica* resembles AMF in its characteristics (Qiang *et al.*, 2011), *C. amaranticolor* might be a non host to *P. indica* resulting in the absence of root colonization.

In case of cowpea, no root colonization was observed with *Fusarium pallidoroseum*. Gokulapalan *et al.* (2006) reported the role of *F. pallidoroseum* in the fascication and yield loss in cowpea. Since *F. pallidoroseum* is pathogenic to cowpea, no symbiotic association of *F. pallidoroseum* might be possible within the cowpea roots resulting in the absence of root colonization.

Co-cultivation of cowpea with *Piriformospora indica* mixed with vermiculite-perlite (3:1) medium at the rate of 1% (w/v) resulted in the root colonization of cowpea with *P. indica* with a percentage root colonization of 40.70 per cent after 14 days of co-cultivation . Vyas *et al.* (2008) reported 85 per cent root colonization of *P. indica* within *Feronia limonia* roots after 30 days of inoculation. Krishnaveni *et al.* (2015) reported the root colonization of *P. indica* in maize with a 55 per cent root colonization. Vyshakhi (2016) reported root colonization of *P. indica* in Chilli, tomato and brinjal as 42.03, 81.81 and 42.03 per cent respectively.

### 5.4.3. Age of the Seedlings and Colonisation

Age of the seedlings study for cowpea with *Piriformospora indica* were conducted in protrays and biometric observations like colonization pattern and

efficiency, root and shoot biomas, number of secondary and tertiary roots, roots hairs per unit area, number of leaves and leaf area were carried out.

An increase in all the above biometric observations were observed in *P. indica* colonized cowpea plants compared to the non colonized cowpea plants. Under the study, colonization pattern was observed using compound microscope and highest efficiency (42.30 per cent) was recorded for five day old seedlings at twenty days after co-cultivation. Root biomass, number of secondary roots, tertiary roots, number of leaves and leaf area showed highest per cent increase for five day old seedlings at twenty days after co-cultivation whereas shoot biomass, root hairs per unit area showed highest per cent increase for seven days old seedlings at twenty days after co-cultivation.

According to Sheramati *et al.* (2008) eighteen day old arabidopsis seedlings when co-cultivated for nine days resulted in a threefold difference in the fresh weight and a more than two fold difference in the chlorophyll content. Nine day old arabidopsis seedlings when transferred to KM media containing *P. indica* fungal lawn showed a two fold increase in shoot and root fresh weight after seven days of co-cultivation (Vahabi *et al.*, 2013).

Hayes *et al.* (2014) reported that *P. indica* inoculated *Medicago truncatula* had a significantly greater number number of roots (102%), total root length (88%), dry weight (25%), heavier shoots (30%), increased leaf area (98%), increased root number (51%) and increased root length (48%) compared to the uninoculated plants after fifteen days of co-cultivation. Sharma *et al.* (2014) reported an overall increase in plant biomass in terms of higher shoot and root length, number of shoots and roots in *P. indica* colonized aloe vera plants. Krishnaveni *et al.* (2015) reported an increase in the growth parameters like fresh weight, dry weight, shoot height, root height and leaf length of maize plants colonized with *P. indica* compared to non colonized maize plants.

*P. indica* colonized plants results in an increase in fresh and dry weights due to enhanced phosphorous uptake (Sudha *et al.*, 1998). In *P. indica* treated plants, the intensified root proliferation may be due to the synthesis of yet unidentified extra cellular phytohormones (Varma *et al.*, 2001). Rai *et al.* (2001) suggested that the differences in the growth observed between *P. indica* treated and control plants might be due to the greater absorption of water and nutrients as a result of extensive colonization by *P. indica*. Kumar *et al.* (2009), reported an increase in biomass production of *P. indica* colonized plants compared with non colonized plants and this increase in biomass was suggested to be due to the mycorrhiza – like growth promoting activity of *P. indica*. The present investigation also showed an increase in all biometric observations.

## 5.5. MANAGEMENT OF BlCMV USING BOTANICALS AND FUNGAL ROOT ENDOPHYTES

### 5.5.1. Bioassay of Botanicals in Local Lesion Host

Various botanicals inducing resistance like *Boerhaavia diffusa, Phyllanthus niruri, Calotropis gigantean, Psidium guajava, Zingiber officinale, Aloe vera* and *Euphorbia hirta* were tested for their effect on BlCMV of cowpea. Pre, simultaneous and post inoculation treatments were done on the local lesion host of the virus, *C. amaranticolor*. These studies on the efficacy of botanicals having antiviral properties found that the spraying of *P. niruri* and *B. diffusa* showed significant inhibition of local lesions when compared to other treatments. *P. niruri* was found effective in pre, simultaneous and post inoculation method of application even at low concentration. In accordance with the present findings, Veena (2007) reported a complete inhibition of CABMV by the *P. niruri* application before inoculation of the virus or when applied along with the inoculum.

Pre inoculation application of plant extracts was found to be more effective than post inoculation application in reducing incidence of *Cowpea mosaic virus* (Mallika Devi, 1990). This reduction in vulnerability of cowpea plants may be due to increased activity of phenols, peroxidase, polyphenol oxidase and phenylalanine ammonialyase (Renukadevi *et al.*, 2004).

Prasad *et al.* (2007) evaluated the efficacy of certain plant extracts in reducing *Bean common mosaic virus* strain *Blackeye cowpea mosaic virus* (BCMV-BlCMV) in cowpea and found that when plant extracts were mixed with BCMV-BlCMV inoculums, a reduction in disease incidence was observed under greenhouse conditions when compared to control. Plant extracts operates induced resistance through the activation of natural defence mechanisms of the host plant and induces systemic resistance to viral multiplication in plants. The active products present in these extracts have no direct effect on viruses; their antiviral activity is mediated by host cells in which they induce the antiviral state. Systemic resistance inducers obtained from plant extracts was also found to be effective against a wide range of viruses (Verma and Baranwal, 2011) .

### 5.5.2. Evaluation of AVP and fungal endophyte treated Cowpea Plants

AVP that showed significant inhibition percentage of the virus was further selected for its assay in cowpea. Among the botanicals inducing resistance, *Phyllanthus niruri* and among the fungal endophytes, *Piriformospora indica* were selected for the study. Vulnerability index was calculated and it was found that *P. niruri* pre treated plants showed least vulnerability to infection followed by *P. indica* pre inoculated plants even at 30 DAI. Concurrent to the present findings, Veena (2007) reported a reduction in the vulnerability of cowpea plants pre treated with *P. niruri* to CABMV compared to the control plants. Achats *et al.* (2010) reported that the *P. indica* induced enhanced growth with improved resistance against biotic and abiotic stress in barley.



The aqueous leaf extracts of *Psidium guajava*, *Phyllanthus niruri* and *Thuja occidentalis* were found to be effective in reducing the infection by *Tomato mosaic virus* (ToMV) (Deepthi *et al*., 2007). Waller (2005) reported that *P. indica* colonized barley plants showed higher resistance to pathogens with an increased yield. Field trials on *Lagenaria siceraria* and *Tagetes sp.* showed that interaction with *P. indica* suppressed the infestation by plant pathogens including viruses (Shrivastava and Varma, 2014).

Some of the evidences showed that in many cases, viral inhibition was due to the development of virus inhibitory substances within the tissues, but some induces systemic resistance (Verma and Prasad, 1984). Prasad *et al.* (2007) reported that the antiviral efficacy may be attributed to the presence of antiviral compounds such as lignin, terpenoids, alkaloids and specific proteins.

## 5.6. Biochemical Changes of Host Pathogen Interaction

### 5.6.1. Estimation of total soluble protein

Estimation of total protein indicated significant difference in the protein level of healthy and BlCMV inoculated plants. In the virus inoculated plants protein content was found to increase with the plant age and recorded maximum at 30 DAI. In healthy plants also protein content increased at different time intervals and recorded maximum at 30 DAI. An increase in protein level in virus infected plants have also been reported by several workers (Chakraborty *et al.,* 1993; Shivaprasad *et al.,* 2005). All these reports are in conformity with the results of the present investigation.

Krishnapriya (2015) reported a significant increase of protein content in BlCMV inoculated cowpea plants without any significant difference of protein content in healthy cowpea plants. Sinha and Srivastava (2010) reported that due to

virus infection, there was an increased demand for abnormal protein production which was required for the rapid synthesis of virus particles. This demand was met upon by the increased diversion of assimilated carbon compounds towards protein synthesis.

Among the treatments, cowpea plants pre inoculated with *P. niruri* recorded maximum protein at 30 DAI (4.60 mg $g^{-1}$). This was followed by cowpea plants pre inoculated with *P.indica.* Veena, 2007 reported a several fold increase in protein content in *P. niruri* pre inoculated cowpea plants compared to healthy control. Manickam *et al.* (2000) studied the impact of application of a foliar spray of AVPs and inoculation of TSWV on total soluble protein contents of cowpea plants. It was found that AVP treated cowpea plants showed marginal increase in protein contents compared to significant increase in TSWV inoculated plants. The increase in total soluble protein in TSWV inoculated plants might be due to the synthesis of virus coat proteins whereas in AVP treated plants, the increased protein content might be due to the formation of new proteins. Vyas *et al.* (2008) reported that *in vitro* raised plantlets of *Feronia limonia* (L.) Swingle when colonized with *P. indica* revealed an increase in total protein content compared to the uncolonized control plants.

Protein profile study was also conducted to analyse the expression of new proteins. The study showed an increase in the protein level from 5DAI to 15 DAI with most prominent induction of novel proteins in cowpea leaves pre inoculated with *P. niruri* followed by cowpea leaves pre inoculated with *P. indica.* Krishnapriya (2015) reported that the BlCMV inoculated cowpea plants showed an increasing trend in the induction of novel proteins with its maximum values at later stages of observation.

### 5.6.2. Estimation of Total Sugars

The present investigation revealed that there was significant difference in the total sugar content between healthy and BlCMV inoculated cowpea plants. The

BlCMV inoculated cowpea plants showed a significant decrease in total sugar content, compared to the uninoculated healthy plants. Concurrent to the findings, Krishnapriya (2015) reported that BlCMV infection in cowpea plants showed a significant decrease in total sugar content when compared to healthy plants. Sindhu (2001) also reported a reduction in the level of carbohydrate content in susceptible varieties inoculated with BlCMV compared to healthy control.

Mali *et al.* (2000) reported that *Mungbean yellow mosaic virus* (MYMV) infection on moth bean resulted in significant reduction of total soluble carbohydrates in susceptible cultivar (GMO 9101) when compared to resistant cultivar (CZM 79). The decrease in carbohydrate can be explained on the basis of faster breakdown of carbohydrates due to increased respiration, followed by conversion of carbohydrates into amino acids (Sinha and Srivastava, 2010).

AVP treated plants showed increased total sugar content compared to control. In cowpea plants pre inoculated with *P. niruri* recorded maximum value at 15 DAI. This was followed by cowpea plants pre inoculated with *P. indica*. Concurrent to the present findings, Veena (2007) reported an increased total sugar content in AVP treated cowpea plants when compared to control. Das *et al.* (2014) revealed an increased sugar content in aerobic rice inoculated with *P. indica* compared to the control ones.

Manickam *et al.* (2000) reported that foliar spray of AVPs from *Cocos nucifera, Sorghum vulgare, Sorghum bicolor* and *Croton sparsiflorus* leaves increased the sugar content in cowpea plants while TSWV inoculation decreased its concentration. Ghabooli (2014) revealed that *P. indica* inoculated barley plants showed an increase in the sugar content compared with the control plants without *P. indica*.

### 5.6.3. Estimation of Reducing Sugar

Reducing sugar content was found to be more in healthy plants when compared to virus inoculated plants. It was found to increase with increase in plant age and reached maximum at 15 DAI and there after it declined. Thind *et al.* (1996) reported that the amount of reducing sugar decreased in black gram infected with *Yellow mosaic virus* when compared to healthy plants.

Cowpea plants pre treated with *P. niruri* showed an increased reducing sugar content compared to healthy and BlCMV inoculated control. This was followed by cowpea plants pre inoculated with *P. indica*. Veena (2007) reported similar results with higher values of reducing sugar content for AVP treated cowpea plants and assumed that the increased sugar content in treated plants might be due to its accumulation as a result of the disruption of normal phloem transport.

### 5.6.4. Estimation of Phenol

The results showed a significant increase in the phenol content of BlCMV inoculated cowpea plants compared to healthy plants. In line with the present findings, Shilpasree *et al.* (2012) reported that BlCMV infection increased the total phenols in diseased cowpea plants compared to healthy ones. Increased level of phenols suggested an accelerated phenol synthesizing pathway following pathogen infection (Meena *et al.*, 2008).

Sutha *et al.* (1997) reported that the accumulation of phenol observed in virus infected plants may be due to excess production of hydrogen peroxide by increased respiration or due to activation of HMP – shunt pathway, acetate pathway and release of bound phenolics by hydrolytic enzymes. Krishnapriya (2015) reported a higher phenol content for BlCMV inoculated cowpea plants compared to healthy plants in case of both resistant and susceptible variety.

Among the treatments, cowpea plants pre treated with *P. niruri* showed higher phenolic accumulation against healthy and BlCMV inoculated control. It was found

to be increased with increase in plant age and reached maximum at 15 DAI and thereafter it declined. In case of cowpea plants treated with *P. indica*, phenolic accumulation continued to increase from 5 DAI to 30 DAI. Veena (2007) reported a multifold increase in the phenolic content of AVP treated cowpea plants compared to healthy and CABMV inoculated plants and explained that the increase may be due to increased phenolic accumulation or due to the excess production of $H_2O_2$ via increased respiration or due to the activation of hexose - monophosphate pathway, acetate pathway and release of bound phenols by hydrolytic enzymes. Sharma *et al.* (2014) reported an increase in total phenol content during the symbiotic association of *P. indica* with aloe vera compared to uninoculated aloe vera plants. An increase in total phenol content was observed in *Pennisetum glaucum* seeds treated with *P. indica* than the control plants (Mishra *et al.*, 2014).

### 5.6.5. Estimation of Peroxidase (PO)

Peroxidase activity was found to be higher in BlCMV inoculated cowpea plants compared to healthy plants. The maximum value was recorded at 15 DAI and thereafter a decline in the value was observed. Sindhu (2001) also reported a significant increase of peroxidase activity in BlCMV inoculated cowpea plants compared to healthy plants. Krishnapriya (2015) reported a higher peroxidase activity for BlCMV inoculated cowpea plants compared to healthy plants. In contrast to the present study, Veena (2007) reported higher peroxidase activity in healthy cowpea plants compared to CABMV inoculated plants.

Application of AVP and endophyte, increased the peroxidase activity. Cowpea plants Pre treated with *P. niruri* recorded maximum peroxidase activity. This was followed by plants pre inoculated with *P. indica*. Veena (2007) reported a similar increase in AVP treated cowpea plants challenged

with BlCMV. Vyas *et al.* (2008) reported an increased peroxidase activity in *P. indica* colonized *Feronia limonia* plants compared to the non colonized plants.

### 5.6.6. Estimation of Polyphenol Oxidase (PPO)

The present study revealed a significantly higher polyphenol oxidase activity in the BlCMV inoculated cowpea plants compared to the healthy plants. In accordance with the results, Sindhu (2001) reported higher values of PPO activity for BlCMV inoculated cowpea plants compared to healthy in susceptible variety Sharika. Krishnapriya (2015) reported a non-significant difference in the inoculated plants of susceptible variety compared to the uninoculated counterparts.

Cowpea plants pre inoculated with *P. niruri* leaf extract recorded higher values compared to healthy and BlCMV inoculated cowpea plants. This was followed by plants pre inoculated with *P. indica*. Similar to the present findings, Veena (2007) reported an increase in PPO activity for AVP treated cowpea plants compared to CABMV inoculated and healthy cowpea plants. Verma and Prasad (1984) found that spraying aqueous leaf extract of *Clerodendron aculeatum* prevented infection of *Sunhemp rosette virus* on cluster beans. The resistance thus induced was due to increased activity of catalase, peroxidase and polyphenol oxidase. At the early time point of co-cultivation of *P. indica* with Arabidopsis, stress and defense-related genes were up-regulated thereby appeared to respond to chemical mediators released by the fungus (Vahabi *et al.*, 2015).

### 5.6.7. Estimation of Phenylalanine Ammonialyase (PAL)

Compared to healthy samples there was an increase in PAL activity in BlCMV inoculated cowpea plants with a decreasing trend from 5DAI to 30 DAI. Umamaheswaran (1996) reported that there was an increase in PO, PPO and PAL activity in inoculated susceptible varieties of cowpea due to CABMV infection. Sindhu (2001) reported a higher PAL activity for BlCMV inoculated cowpea plants compared to healthy in susceptible variety Sharika. Krishnapriya (2015) reported a significant increase in the inoculated resistant genotypes compared to the uninoculated and susceptible genotype throughout the period of observation with a decreasing trend from 5DAI. According to Veena (2007) the decreasing trend in the PAL activity may be due to the susceptibility of the plants to the virus.

Application of AVPs, increased the accumulation of PAL, when challenged with BlCMV in cowpea plants compared to both healthy and virus inoculated control. Among the treatments, cowpea plants pre inoculated with *P. niruri* was found to perform better followed by plants pre treated with *P. indica*. Veena (2007) reported a higher PAL activity for cowpea plants pre treated with *P. niruri* and assumed that the increase in the activity may be due to the level of variation in the signal molecules that elicited a cascade response, *via,* activating the series of defence response against viral infection. Among fourteen days of co-cultivation, a strong difference in the response of defence enzymes were observed before six days and later no remarkable changes were observed in *P. indica* colonized Arabidopsis plants (Vahabi *et al.*, 2015).

### 5.6.8. Electrophoretic Analysis of Proteins by SDS-PAGE

Electrophoretic analysis of proteins was done to elucidate the effect of different treatments to the plant – virus interactions and consequent induction of novel proteins. Expression of new proteins were analysed and several defence related

proteins and enzymes seems to be involved in the inhibition of BlCMV in cowpea as a result of various treatments. Many novel proteins were induced during the initial stages of treatments and induction of proteins increased from 5DAI to 15DAI and later declined.

The induction of novel proteins was more prominent in cowpea leaves pre inoculated with *P. niruri* which was followed by cowpea leaves pre treated with *P. indica*. The induction of defence proteins and enzymes by various biotic and abiotic factors were well established in inhibition of viral diseases in plants (Deverall and Dann, 1995; Mali *et al.*, 2000). Veena (2007) reported a decreasing trend in the induction of novel proteins with maximum protein induction for plants treated with biotic and abiotic factors compared to that of healthy and inoculated control. Krishnapriya (2015) reported an increasing trend in the induction of novel proteins in BlCMV inoculated cowpea plants with its maximum values at later stages of observation.

# *Summary*

## 6. SUMMARY

Economically significant and cosmopolitan seed borne virus of cowpea causing mosaic disease in cowpea was selected for the study owing to their widespread occurrence and heavy yield loss among the cultivars in Kerala. The present study entitled "Exploration of natural products from botanicals and fungal root endophytes for the management of *Cowpea mosaic virus*" was carried out at Department of Plant Pathology, College of Agriculture, Vellayani during 2014-2016, with the objective to develop serological and molecular tools for the early detection of *Cowpea mosaic virus*; and its management using natural products from botanicals and fungal root endophytes.

*Cowpea mosaic virus* was maintained in major host cowpea (*Vigna unguiculata* var. *sesquipedalis* (L.) Verdcourt) variety Sharika and local lesion host *Chenopodium amaranticolor* by mechanical inoculation. On the newly emerged trifoliate leaves of cowpea, the initial symptoms appeared as vein clearing. Typical symptoms appeared as dark green and light green mottling, mosaic, vein banding, leaf distortion and stunting of plants with reduced pod production. On the mechanically inoculated *Chenopodium amaranticolor* leaves, localized yellow chlorotic lesions were observed five days after inoculation.

Fungal root endophytes *Piriformospora indica* and *Fusarium pallidoroseum* were maintained by continuous subculturing in Potato dextrose agar medium. The *P. indica* produced nine cm radial growth on nine DAI whereas *F. pallidoroseum* produced nine cm radial growth on five DAI in Petri dishes.

Immunological detection of the mosaic causing virus in cowpea was done using Direct antigen coating – Enzyme linked immunosorbent assay (DAC-ELISA) and Dot immunobinding assay (DIBA). In DAC-ELISA, the polyclonal antibody (H2) against BlCMV and polyclonal antibody (H4) against CABMV gave a positive

reaction in samples collected from symptomatic plants. The monoclonal antibodies (15E6, 16G5) against BlCMV gave a positive reaction in samples collected from symptomatic plants while it did not react with the monoclonal antibodies (7A10, 5H5) of CABMV indicating the mosaic causing virus in cowpea as BlCMV.

DIBA using polyclonal antibodies against BlCMV (H2) and CABMV (H4) gave positive reaction for both with highest mean value of intensity for H2 indicating the presence of BlCMV as the mosaic causing virus in cowpea.

Molecular detection *viz.* reverse transcription-polymerase chain reaction (RT-PCR) using BlCMV specific primers along with the primers already published, produced amplicons of sizes ~ 425 bp and ~ 110 bp respectively. CABMV specific primers designed and that obtained from published papers didnot show any amplification, thus confirming the presence of BlCMV as the mosaic causing virus in cowpea.

Standardization of co-cultivation method for the fungal root endophytes with *C. amaranticolor* and cowpea were conducted in jam bottles but no colonization of any fungal endophytes were observed in both *C. amaranticolor* and cowpea. Colonization studies in vermiculite-perlite (3:1) medium at the rate of 1% (w/v) conducted in protrays showed no colonization during the co-cultivation studies of *F. pallidoroseum* with both cowpea and *C. amaranticolor*. In case of *P. indica*, no colonization was observed for *C. amaranticolor* whereas in cowpea, after 7 days of co-cultivation chlamydospores were observed on the surface of the roots and no colonization was observed inside the roots. But colonization of fungus was observed within the roots after 14 days of co-cultivation of cowpea with *P. indica* with a per cent root colonization of 40.70 per cent.

In a study conducted to determine the effect of age of seedlings on colonization pattern was observed using compound microscope and the highest efficiency (42.30 per cent) was recorded for five day old seedlings at twenty days

after co-cultivation. Highest values for per cent increase of root biomass (77.77 per cent), number of secondary roots (73.54 per cent), tertiary roots (91.46 per cent), number of leaves (42.01 per cent) and leaf area (62.55 per cent) was observed for five day old seedlings at twenty days after co-cultivation whereas shoot biomass (80.68) and root hairs per unit area ( 70.17 per cent) showed highest values for seven day old seedlings at twenty days after co-cultivation.

Seven botanicals having antiviral properties were screened to find out their efficiency in local lesion host of the virus, *Chenopodium amaranticolor*. Among the seven botanicals, *Phyllanthus niruri* recorded more than 80 per cent inhibition of local lesions in *C. amaranticolor* even at one per cent concentration. Highest inhibition (94.84 per cent) was observed for pre inoculation application of *P. niruri* at five per cent concentration.

The best antiviral principle (AVP) and fungal endophyte *viz*., *P. niruri* and *P. indica* respectively were evaluated in cowpea against BlCMV. A reduction in vulnerability towards BlCMV was observed in cowpea plants pre inoculated with *P. niruri* (one per cent) and *P. indica* (one per cent) at all intervals of observation compared to that of the virus inoculated control plants.

The biochemical changes due to virus infection was studied in selected treatments, virus inoculated and healthy cowpea plants. There was a progressive increase in the total protein content both in virus inoculated and healthy plants with increase in the age of the plant. Among the treatments pre inoculation treatment of *P. niruri* recorded maximum protein content. Analysis of total sugar content revealed that the highest values of sugar content was in healthy and this was followed by cowpea plants pre inoculated with *P. niruri*. *P. niruri* and *P. indica* treated cowpea plants challenged with BlCMV induced higher accumulation of phenolics when compared to virus inoculated and healthy cowpea plants. Studies on defence related enzymes *viz.,* peroxidase, polyphenol oxidase and phenyl alanine revealed that the cowpea plants pre inoculated with *P. niruri* showed the highest activity of these

enzymes which was followed by cowpea plants pre treated with *P. indica*. Electrophoretic analysis of proteins revealed that many novel proteins were induced in response to various treatments. The induction of novel proteins was more in leaves treated with *P. niruri* and *P. indica* than the control plants. Novel proteins (45, 43, 21, 31, 27, 30, 97.4, 21, 20.5, 78, 86, 97.4, 54, 20.1, 22, 25, 27, 29, 35, 37, 52, 29,35,26, 27, 66, 18 and 26 KDa) were induced in response to *P. niruri* treatments. New proteins (45, 43, 27, 30, 97.4, 14.3, 20.1, 78, 86, 20.1, 22, 25, 29, 52, 20.1, 52, 18, 26, and 29 KDa) were observed in response to *P. indica* treatments.

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# EXPLORATION OF NATURAL PRODUCTS FROM BOTANICALS AND FUNGAL ROOT ENDOPHYTES FOR THE MANAGEMENT OF *Cowpea mosaic virus*

*by*

**THERESA ALEX**

**(2014-11-133)**

**Abstract of the thesis**

**Submitted in partial fulfilment of the**

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**DEPARTMENT OF PLANT PATHOLOGY**

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**KERALA AGRICULTURAL UNIVERSITY**
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DEPARTMENT OF PLANT PATHOLOGY

**DEFENSE SEMINAR**

**ABSTRACT**

**THERESA ALEX**
**2014-11-133**

**DATE: 10-11-2016**
**TIME: 9:15-10:00 AM**

The present study entitled "Exploration of natural products from botanicals and fungal root endophytes for the management of *Cowpea mosaic virus*" was carried out at Department of Plant Pathology, College of Agriculture, Vellayani during 2014-2016, with the objective to develop serological and molecular tools for the early detection of *Cowpea mosaic virus*; and its management using natural products from botanicals and fungal root endophytes.

*Cowpea mosaic virus* was maintained in local lesion host *Chenopodium amaranticolor* and cowpea (*Vigna unguiculata* var. *sesquipedalis* (L.) Verdcourt) variety Sharika by mechanical inoculation. On the newly emerged trifoliate leaves of cowpea, typical symptoms like mosaic and vein banding followed by leaf distortion were expressed seven days after inoculation (DAI). In *C. amaranticolor,* symptoms were expressed five DAI and on the inoculated leaves, localized yellow chlorotic lesions were observed. Fungal root endophytes *Piriformospora indica* and *Fusarium pallidoroseum* were maintained by continuous subculturing in Potato dextrose agar medium. The *P. indica* produced nine centimetre radial growth on nine DAI whereas *F. pallidoroseum* produced nine centimetre radial growth on five DAI in petri dishes.

Serological studies carried out using Direct antigen coating – Enzyme linked immunosorbent assay (DAC-ELISA) and Dot immunobinding assay (DIBA) and molecular detection *viz.*, Reverse transcription-polymerase chain reaction (RT-PCR), identified the mosaic causing virus in cowpea as *Blackeye cowpea mosaic virus* (BlCMV).

Botanicals for the management of BlCMV were first screened on *C. amaranticolor* to find out their efficiency. Among the seven botanicals, *Phyllanthus niruri* recorded more than 80 per cent inhibition of local lesions in *C. amaranticolor* even at one per cent concentration. Highest inhibition (94.84 per cent) was observed for pre inoculation application of *P. niruri* at five per cent concentration.

Standardization of co-cultivation method for the fungal root endophytes with *C. amaranticolor* and cowpea were conducted in jam bottles but no colonization of any fungal endophytes were observed in both *C. amaranticolor* and cowpea. Colonization studies in vermiculite-perlite (3:1) medium at the rate of 1% (w/v) conducted in protrays showed no colonization during the co-cultivation studies of *F. pallidoroseum* with both cowpea and *C. amaranticolor*. In case of *P. indica*, no colonization was observed for *C. amaranticolor* whereas 40.70 per cent root colonization was recorded in cowpea.

In a study conducted to determine the effect of age of seedlings on colonization pattern observed using compound microscope, the highest efficiency (42.30 per cent) was recorded for five day old seedlings at twenty days after co-cultivation. Root biomass, number of secondary roots, tertiary roots, number of leaves and leaf area also showed high values for five day old seedlings at twenty days after co-cultivation whereas shoot biomass and root hairs per unit area showed highest values for seven day old seedlings at twenty days after co-cultivation.

The best antiviral principle (AVP) and fungal endophyte *viz.*, *Phyllanthus niruri* and *Piriformospora indica* respectively were evaluated in cowpea against BlCMV. Vulnerability of cowpea plants to BlCMV was found to reduce with *P. niruri* and *P. indica* treatments compared to that of control plants.

The biochemical analysis to study the mechanisms involved revealed that cowpea plants pre inoculated with *P. niruri* was found to be superior followed by pre inoculation treatment with *P. indica* except for total sugar where maximum values for healthy was followed by pre inoculation treatment with *P. niruri*. Many

defense and pathogenesis related (PR) proteins were induced in response to various treatments. The induction of novel proteins were more in cowpea leaves which were treated with *P. niruri* and *P. indica* than in control plants.

Thus it is proved by serological and molecular detection methods that the virus causing mosaic disease in cowpea is BlCMV. For the management of this virus pre inoculation treatment of *P. niruri* (extract of one gram sample in 100 ml water) and pre inoculation treatment of *P. indica* (one gram mycelia in 100 ml vermiculite-perlite media) has been proved to be effective. It can be further validated under field condition.

# *Appendices*

## APPENDIX - I

### Buffers for sap extraction

**1. 0.1M Sodium phosphate buffer (pH 7.0)**

Stock solutions

A: 0.2 M Solution of monobasic sodium phosphate (27.8 g in 1000 ml)

B: 0.2 M Solution of dibasic sodium phosphate (53.65 g of Na2.HPO4.12H2O in 1000ml)

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

## APPENDIX - II

### Potato Dextrose Agar (PDA) Medium

| | |
|---|---|
| Potato | : 200 g |
| Dextrose | : 20 g |
| Agar-agar | : 20 g |
| Distilled water | : 1 litre |

## APPENDIX - III

### Buffers for DAC-ELISA

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

| | | |
|---|---|---|
| Sodium chloride | - | 8.0 g |
| Potassium dihydrogen phosphate | - | 0.2 g |
| Disodium hydrogen phosphate | - | 1.1 g |
| Potassium chloride | - | 0.2 g |
| Sodium azide | - | 0.2 g |
| Water | - | 1000 ml |

**2. Wash Buffer**

Add 0.5 ml/L of Tween-20 to PBS

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

| | | |
|---|---|---|
| Sodium carbonate | - | 1.59 g |
| Sodium bicarbonate | - | 2.93 g |
| Sodium azide | - | 0.2 g |
| Water | - | 1000 ml |

**4. Antibody diluents buffer**

Add 20 g PVP and 2 g ovalbumin to 1 L PBS-T

**5. Enzyme conjugate diluents buffer (PBS-TPO)**

Same as PBS-TPO

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

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

Add HCl to attain the required pH (9.8).

## APPENDIX - IV

## Stock Solutions for DIBA

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

   0.02 M Tris - 4.84 g

   0.5M NaCl - 58.48 g

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

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

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

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

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

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

   Same as TBS-SDM

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

   0.1 M Tris - 12.11 g

   0. 1 M NaCl - 5.85 g

   5mM $MgCl_2.6H_2O$ - 1.01 g

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

**6. Substrate solution**

Solution A

Nitro blue tetrazolium (NBT) - 75 mg

Dimethyl formamide (MFA) - 1 ml

Solution B

Bromo chloro indolyl phosphate(BCIP) - 50 mg

DMFA - 1 ml

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

**7. Fixing solution (pH 7.5)**

10 mM Tris - 1.21 g

1 mM EDTA - 0.29 g

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

## APPENDIX - V

### Buffers for PCR Analysis

**1. 50 X TAE buffer (Tris-Acetate-EDTA) (pH 8.0)**

Tris base - 242.0 g

Acetic acid - 57.1 ml

0.5 M EDTA - 100 ml

Add distilled water to a final volume of 1 litre.

**2. Sample loading buffer (6x)**

0.25% Bromophenol blue

40% (w/v) sucrose in water

**3. Master mix for cDNA synthesis**

| | | |
|---|---|---|
| Buffer (5x) | - | 4 µl |
| dNTP mix (10mM each) | - | 2 µl |
| Oligo dT (20 µm) | - | 1 µl |
| Long range RNAase inhibitor | - | 0.2 µl |
| Long range reverse transcriptase | - | 1 µl |
| RNAase free water | - | variable |
| Template RNA | - | variable |
| Total volume | - | 20 µl |

## APPENDIX VI

### Plant Nutrient Medium (PNM)

$KNO_3$ - 5Mm

$MgSO_4.7H_2O$ - 2mM

$Ca(NO_3)_2$ - 2mM

Fe-EDTA - 2.5 ml

Micronutrient mix - 1.0 ml

Agar - 10.0 g

## APPENDIX VII

### ESTIMATION OF PROTEIN

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

Stock solutions

A: 0.2 M solution of Acetic acid (11.5 ml in 1000 ml)

B: 0.2 M solution of Sodium acetate (16.4 g of $C_2H_3O_2Na$ or 27.2 g of $C_2H_3O_2Na.3H_2O$ in 1000 ml)

22.7 ml of A is mixed with 27 ml of B, diluted to a total of 100 ml.

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

100 mg of Coomassie brilliant blue G-250 was dissolved in 50 ml of 95% ethanol and 100 ml of concentrated Orthophosphoric acid was added. The volume was made up to 200 ml with water and kept at $4^0$C. The working dye was prepared just before use by diluting the stock solution to five times with water.

## APPENDIX VIII

## ESTIMATION OF Total Sugars

1. **Anthrone reagent**

Anthrone reagent made by dissolving 200 mg of anthrone in 100 ml of ice cold 95% concentrated Sulphuric acid.

## APPENDIX IX

## ESTIMATION OF reducing Sugars

1. **Dinitrosalicylic acid reagent**

| | | |
|---|---|---|
| Dinitrosalicylic acid | : | 1 g |
| crystalline phenol | : | 200 mg |
| Sodium sulphite | | 50 mg |
| 1 % sodium hydroxide | : | 100 ml |

## APPENDIX X

## BUFFERS FOR ENZYME ANALYSIS

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

   Stock solutions

   A: 0.2 M Solution of monobasic sodium phosphate (27.8 g in 1000 ml)

   B: 0.2 M Solution of dibasic sodium phosphate (53.65 g of $Na_2.HPO_4.12H_2O$ in 1000 ml)

   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 solution Boric acid (12.4 g in 1000 ml)

   B: 0.05 M solution of Borax (19.05 g in 1000ml)

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

## APPENDIX XI

## ELECTROPHORETIC ANALYSIS OF PROTEINS USING SDS-PAGE

**1. Protein denaturing solution**

10 M urea - 80 ml

1 M $NaH_2PO_4.2H_2O$ (pH 8) - 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 | - | 100.0 ml |

**3. Separating (resolving) gel buffer stock (1.5 M Tris-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 6 N HCl and volume was made up to 100 ml with double distilled water and stored at $4^0$C.

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

Tris base (6.0 g) was dissolved in approximately 60 ml of double distilled water. The pH was adjusted to 6.8 with 6 N HCl and the volume was made up to 100 ml with double distilled water and stored at $4^0$C.

**5. Polymerising agents**

Ammonium per sulphate (APS) – 10 % prepared freshly before use.
TEMED – fresh from refrigerator

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

| | | |
|---|---|---|
| Tris base | - | 6.0 g |
| Glycine | - | 28.8 g |
| SDS | - | 2.0 g |
| Double distilled water | - | 2 L |

**7. Sample buffer**

| | | |
|---|---|---|
| Double 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 |
| Double distilled water | - | 50.0 ml |

**9. Destaining solution**

As above without Coomassie brilliant blue R 250.

**10. Preparation of separating gel (12%)**

| | |
|---|---|
| Double distilled water | - 6.7 ml |
| Tris HCl (pH 8.8) | - 5.0 ml |
| SDS 10 per cent | - 0.2 ml |
| Acrylamide stock | - 8.0 ml |

The above solution was mixed well and degassed for 3 min and then the following were added immediately.

| | |
|---|---|
| 10% freshly prepared Ammonium per sulphate (APS) | - 0.10 ml |
| Tetra methyl ethylenediamine (TEMED) | - 0.01 ml |

The separating gel was mixed well and poured immediately between glass plates and a layer of water was added above the polymerizing solution to quicken the polymerization process.

**11. Preparation of stacking gel (4%)**

| | |
|---|---|
| Double distilled water | - 6.1 ml |
| Tris HCl (pH 6.8) | - 2.5 ml |
| SDS 10 per cent | - 0.2 ml |
| Acrylamide stock | - 1.3 ml |

The solution was mixed well, degassed and the following were added.

| | |
|---|---|
| APS 10% | - 0.05 ml |
| TEMED | - 0.1 ml |