Chapter 5
DISCUSSION

Muga food plants have wide distribution throughout the North-Eastern India and in some parts of Northern India. These plants are widely available in the states of Assam, Meghalaya, Manipur, Mizoram, Nagaland, Tripura, Sikkim, Himachal Pradesh, Uttaranchal, Uttar Pradesh, Gujarat, West Bengal and Pondicherry and sporadically available in Arunachal Pradesh. It was also reported that they are present in Nepal, Myanmar, Malaysia, Indonesia, Bhutan and Srilanka (Tikader and Kamble, 2010). All the muga host plants grow well in wet and warm climatic conditions with high rainfall and soil pH range of 4.0- 6.8. Muga silkworm host plant, som (*Persea bombycina* Kost.) is a heterogeneous wild deciduous tree available abundantly in natural forest of northeast India. It exhibits variability in several desirable and undesirable traits.

Although, collection of muga plant genetic resources was initiated in 1988 by the Regional Muga Research Station (RMRS), Boko, Kamrup, Assam, only fourteen cultivars of som and ten cultivars of soalu could be collected from Assam and Meghalaya. After initial evaluation, 8 som and 10 soalu genotypes were selected for further studies. These genotypes were characterized based on morphology, floral biology, propagation, chemo-assay and bioassay (Hazarika*et al*., 1996; Paliwal andDas, 1989; Raja Ram, 1998; Siddiqui *et al*., 1998, 2000; Singh *et al*., 2000; Thangavelu*et al*., 2005). In another effort, 39 som genotypes were collected by the Central Muga Eri Research and Training Institute, Lahdoigarh, Assam. These genotypes were characterized based on a set of characters viz. Plant height(m), No. of branches, Inter nodal distance (cm), Laminar length (cm), Laminar width (cm), no. of leaves and Leaf yield/plant (kg).In general, som trees have the potential of yielding 22-24 MT /ha/year leaf but presently only 16-18 MT/ha/year is obtaining. So, there is an yield gap of more than 8MT/ha/year. To fill up this gap, agronomic and cultural practices need to be improved.

On the basis of leaf size and shape, stem, inflorescence, colour of the sprouted leaves and size of the plants of som, Raja ram *et. al.* (1993) described eight morphotypes of som and named them as Som cultivars -S-1, S-2, S-3, S-4, S-5, S-6,

S-7 and S-8. Kataky and Hazarika (1996), however, categorized the som plant into three groups viz. most preferred, preferred and least preferred based on feeding performance of *A. assama.* On the basis of bio-chemical analysis, bioassay and post cocoon parameters Siddiqui *et al* (2000) reported that S-6, S-4, S-3 and S-5 were palatable and superior for sustainable yield of muga cocoon, whereas three morpho variants viz. S-l, S-2 and *S*-7 were less preferred by the muga silkworm. Therefore in the present study eight different Som morphotypes viz, S1, S2, S3, S4, S5, S6, S7 and S8 were taken into consideration for different experimental work.

Jolly *et al*., (1976) reported that nutritionally rich food plants are required for healthy growth of silkworm. Production of muga silk depends upon the quality of leaves. The nutritional value of leaf, however, varies greatly owing to a number of factors among which the diseases play an important role (Dandin *et al.,* 2003). Being a perennial plant, som plants are exposed to different environmental vagaries and pathogens prevailing in the region throughout the year. As a result, fungi cause number of diseases (Das *et al.,* 2003). Babulal *et al.,*(2000) reported that a considerable amount of leaf yield is lost regularly due to foliar diseases in som plant. Feeding of such infected leaves yielded poor Effective Rate of Rearing (ERR). An extensive survey of literature revealed that a limited quantum of work has been done on pathological aspects i.e. eco-pathology, epidemiology and management of diseases of som plant *(Persea bombycina).*

The present study deals with two major foliar fungal diseases of som plant, Leaf blight and Grey blight. Das and Benchamin (2000) reported that 13.8% - 33.5% leaf yield loss is caused by leaf blight, leaf spot, red rust and leaf curl diseases of som plant. Das *et al* (2005) first reported the occurrence of leaf blight in som plants caused by *Colletotrichum gloeosporioides* that caused approximately 6.3% of the total yield loss in leaf yield. Bharali (1969) and Das and Bechamin (2000) reported Grey blight caused by *Pestalotiopsis disseminata* as a major epidemic disease of muga host plant, som causing 13.8% - 41.6% leaf yield loss. Similarly in the present investigation it was observed that under nursery condition leaf blight was prominent from month of April to August and was highest in S6 morphotype causing 68.2% PDI. On the other hand establishment of grey blight disease was highest in S5 morphotype causing around 72% of disease incidence.

At the outset of the present study symptamology and etiology of the leaf blight and grey blight disease of som plant was studied. Das *et al* (2010) reported the

symptoms and the epidemiological parameters that causes the grey blight disease of som plant. It was recorded that young leaves (leaf no. 1-4 from the top) are more susceptible to the disease than the older leaves. This is in accordance with the results recorded in the present study where the young leaves were more prone to disease and the disease symptoms clearly indicate grey blight disease. It was also discussed that sporulation of *P. disseminata* spores were optimum at 25±2°C and it started at 8h of incubation and increased until 20h of incubation. Conidial morphology is the most widely used taxonomic character for the genus *Pestalotiopsis*. Colour of the median cells is still a widely used character, and all species separate into three groups based on this- concolorous, versicolorous umber olivaceous and versicolorous fuliginous olivaceous. Molecular evidence indicates that it is more precise to group species according to concolorous and versicolorous rather than the above three groups (Jeewon *et al*., 2003).The length of the apical appendages and the number of the apical appendages are also widely used characters for species identification. Some species can also be identified by the presence of knobbed apical appendages. The apical appendages can arise from the top, middle, bottom or different positions in the apical hyaline cells and such characters are widely used in species identification. Furthermore the apical appendages can be divided into branches; in some species presence or absence of the basal appendages is another character for species diagnosis (Maharachchikumbura *et al*; 2011). Based on these different taxonomic classifications, the conidial morphology of the fungal isolates of grey blight was studied and they could be placed under *P. disseminata* group.

On the other hand the growth and sporulation of *Colletotrichum gloeosporioides* causing leaf blight disease of som plant was studied. Chakraborty *et al* (1995a) studied the factors influencing spore germination, appressoria formation and disease development in *Camellia sinensis* by *Glomerella cingulata*. They reported that spore germination and appressoria formation were optimum at a temperature of 25°C, pH 5.0 and a 24h incubation period. In the present investigation optimum condition necessary for growth and sporulation of *Colletotrichum gloeosporioides* was tested in vitro. Maximum growth occurred in Potato Dextrose Agar medium at an incubation period of 10days. Sporulation behaviour and appressoria formation varied with the different media on which the fungus was grown.

In the present study varietal resistance of eight different morphotypes of som plants, against the fungal pathogens *P.disseminata* and *C. gloeosporioides* was carried

out by detached leaf and whole plant inoculation techniques. Responses exhibited by the morphotypes were essentially the same in both the techniques. Of the 8 morphotypes, S5 and S6 morphotypes were more susceptible to the fungal diseases. Chakraborty *et al* (1995b) tested the pathogenecity of three different isolates of *Pestalotiopsis theae* on 12 tea varieties with detached leaf inoculation technique to reveal the susceptible and resistance variety of tea to grey blight disease. Chakraborty *et al* (1996a) also tested pathogenecity of *Glomerella cingulata* towards tea varieties using both detached leaf and cut shoot method.

Studies have been undertaken for detection of fungal pathogens in host tissues by immunological methods by Chakraborty and Chakraborty (2003). The development of serological techniques has produced a number of highly sensitive methods for identifying microorganisms in diseases plant tissues. These rely on solid or soluble antigenic materials by antibodies raised against the organisms and subsequent use of an enzyme labelling system. The possible involvement of cross reactive antigens (CRA) in determining the degree of compatibility has been reported by several workers in different host-pathogen systems, viz., potato-*Phytophthora infestans*(Alba and De Vay, 1985), soybean – *Macrophomina phaseolina* (Chakraborty and Purkayastha, 1983), tea – *Bipolaris carbonum* (Chakraborty and Saha, 1994), groundnut - (Purkayastha and Pradhan, 1994), tea- *Ustulina zonata* (Chakraborty *et al.*, 2002b) and tea – *Exobasidium vexans* (Chakraborty *et al.*, 2009).
In the present study serological relationship between som morphotypes and the two different fungal pathogens, *P. disseminata* and *C. gloeosporioides* were determined using indirect immunofluorescence and indirect immunogold labelling techniques. Cross sections of healthy som leaves exhibited a natural bright yellow autofluorescence mainly on the cuticle. PAb-Cg was most reactive with leaf sections of S6 morphotype. On the other hand leaf sections that were treated with PAb-Pt were more reactive with S5 morphotype. Here reaction with FITC developed fluorescence that was distributed throughout the leaf tissues. The cellular location of CRA in tea leaves shared by *Pestalotiopsis theae* (Chakraborty *et al*., 1995b), *G.cingulata* (Chakraborty *et al*., 1996a) and *E. vexans* (Chakraborty *et al*., 2009) has been studied by immunofluorescent techniques.

Effectiveness of PAbs raised against pathogen was confirmed by immunodiffusion. IgGs were purified before application in immunoenzymatic assays to minimize non-specific binding. Optimization of ELISA was done considering two

variables – dilution of antigen and antiserum. The PTA-ELISA format was employed for the detection of pathogen in artificially inoculated som morphotypes using PAb-Cg and PAb-Pt. Absorbance value (A_{405}) was always higher in infected leaf extracts than healthy ones thereby allowing easy and early detection of infection, as early as 24 hrs of inoculation. Chakraborty *et al* (2009) reported that PTA-ELISA format could easily detect pathogen *Exobasidium vexans* in susceptible variety of *Camellia sinensis* (AV-2) as early as 24h after artificial inoculation whereas the disease symptoms were not visible before 12 days. The results of PTA ELISA was confirmed by Dot-Immunobinding Assay in which intensity of dots widely varied among different morphotypes of som plants artificially inoculated with the pathogens. Early and rapid diagnosis of red rot disease in sugarcane caused by *Colletotrichum falcatum* was also performed using DIBA technique where infected samples depicted dark blue precipitate on the nitrocellulose membrane due to the antigen-antibody reaction (Hiremath *et al*., 2004). Early detection of grey blight pathogens in som morphotypes using PTA-ELISA format and DIBA has already been reported by Acharya *et al* (2015a).

Effectiveness of mycelial antigen of pathogens in raising antibodies was assessed using DIBA. Development of deep violet colour following homologous reaction with antigen and antibody confirmed its identity. Western blot analysis using PAb-Pt and PAb-Cg was also carried out in the present study to develop strategies for rapid detection of pathogens. Here the bands on SDS-PAGE gel were compared with bands on nitrocellulose membrane. Bands of varying molecular weights were seen in SDS-PAGE out of which some bands were also seen on nitrocellulose membrane suggesting these to be the respective epitopes of the antibodies. Chakraborty *et al* (2012) raised polyclonal antibodies against mycelial antigens of *Macrophomina phaseolina* and further used them in immunological formats such as immunodiffusion, PTA-ELISA, dot immunobinding assay, Western blot analysis and indirect immunofluorescence for quick and rapid detection of the pathogen.

In the present investigation indirect immunofluorescence study of young mycelia and fungal spores was carried out with homologous antibody labelled with FITC. Strong apple green fluorescence was seen in both mycelia and spores which confirmed homologous reaction of the pathogen and antibody. The present study also reports the use of indirect immunofluorescence tests using PAb-Cg and PAb-Pt as a suitable technique for localization of the pathogen and could be employed for

immunodetection of pathogen in som leaf tissues. Kratka et al (2002) reported the use of a polyclonal antibody IgG K91 to detect a quarantine pathogen of strawberry, *Colletotrichum acatatum* using four different immunotechniques, PTA-ELISA, dot-blot, immunoprint and immunofluorescent microscopy.

In the current investigation, an attempt was also made to conduct ultrastucturalimmunocytochemical studies to locate cross reactive antigens shared by *Persea bombycina* and its fungal pathogens through transmission electron microscopy (TEM). Encouraging results were obtained following immunogoldcytochemical staining of ultrathin som leaf sections. Electron microscopic observations of healthy leaf tissues treated with PAb-Cg and PAb-Pt showed specific localization of antibody associated with epidermal and mesoplhyll cells. In addition, immunogold labelling of *P. disseminata* and *C. gloeosporioides* in infected som leaf tissues was also performed and intense labelling was observed on fungal mass that was established inside the tissue.

The polymerase chain reaction is undoubtfully the most important technique in diagnostics and has found wide application as a powerfulmolecular tool mostly due to the development of thermotolerant DNA polymerases and automated thermocyclers.PCR is preferred over classical or other moleculartechniques in the diagnosis of plant pathogens for a number of advantages that makes it very popular. Since high quality of DNA is not generally required, there is no needfor culturing the target pathogen. PCR cycles are completedin much shorter time than other molecular techniques, thusallowing a very fast screening of a large number of samples.Because of its high sensitivity, minute amounts of the target DNA are required. The ribosomal DNA gene cluster (rDNAs) is an extensively used target sequence for PCR detection of fungal plant pathogens because of a number of useful features.rDNAs bear common sequences found in the nucleus and themitochondria of eukaryotes. The nuclear rDNA cluster ispresent as tandem repeats of several hundred copies in cell, which allows high sensitivity of detection. The rDNA geneis consisted of three subunits: a large (LSU) of 28S and asmall (SSU) of 18S that are separated by a much smallergene of 5.8S. The three subunits are connected together withtwo internal transcribed spacers (ITS1 and ITS2). This whole gene cluster is repeated in the genome many timesthus being an appealing target for PCR amplification (Paplomatas *et al*., 2006). ITS sequences have gained popularity for being more variable regions and therefore allow selectivedetection of closely related organisms. Universal

primersdesigned on conserved sequences found on the small andlarge subunits, have been extensively used for the amplification of ITS regions. The amplified sequences arebetween 500-800 bp, a relatively small amount of target DNA is required for PCR, while the PCR products have been used as species-specific probes (Bruns *et al*.,1992; Gardes and Bruns,1993; White *et al*., 1990).Moreover, determination of ITS sequences afteramplification by universal primers, has allowed thedetection, identification and taxonomy of unculturable orunknown fugal species.

In the present study, ITS regions of ribosomal genes for construction of primers were used to identify *C. gloeosporioides* and *P. disseminata*. ITS region of rDNA of the pathogens was amplified using genus specific ITS1 and ITS4 primers. Amplified products of size in range of 550bp were produced by the primer pairs. These PCR products were used for sequencing of 18S rDNA region of both the pathogens. The sequence information of the pathogens was then analysed through BLASTn program one at time. The information generated for *C. gloeosporioides* isolates indicated that the sequences contain the genetic information of internal transcribed spacer region of rDNA gene of *C. gloeosporioides* with 100% similarity. On the other hand the sequence information of *P. disseminata* isolates analysed using BLAST n program indicated that the sequences contained genetic information of internal transcribed spacer region of rDNA gene of *Pestalotiopsi*s sp. with 100% similarity.

Identified *C. gloeosporioides* rDNA gene sequences obtained from NCBI Genbank of various host plants were selected for comparisons of rDNA gene sequences of *C.gloeosporioides* isolate of som plant. Similarly, identified different *Pestalotiopsis*r DNA gene sequences obtained from NCBI Genbank of various host plants were also selected for comparison of rDNA gene sequence of *Pestalotiopsis* sp isolate of som plant. Phylogenetic tree was constructed separately for the two different pathogens of som plants to infer the evolutionary history of these isolates. Maharachchikumbura *et al* (2011) discussed that due to overlapping morphological charecters between the different species of *Pestalotiopsis* genus, identification to the species level is rather difficult. For this reason, naming of species is difficult and highly subjective and many sequences for *Pestalotiopsis* deposited in GenBank are likely to be wrongly named. Therefore the Pestalotiopsis isolate in the present investigation could be identified at the genus level only using the molecular identification tools.

On the other hand the present study also conducted specific characterization of *C. gloeosporioides* isolates with primers CgInt-ITS4. The amplification using this primer pair was positive for all the *C. gloeosporioides* isolates of som plant, generating fragments of approximately 450bp and negative for all the *Pestalotiopsis*sp isolates. The use of these primers confirmed the identity of the isolates of *C. gloeosporioides* identified by traditional method. Mills *et al* (1992), Freeman *et al* (2000), Maciel *et al* (2010) and Chowdappa *et al* (2012) used these markers for identification of the *C. gloeosporioides* isolates which showed fragments of 450-bp. The results were similar to those obtained in the present study.

In the present investigation amplified fragments of *Colletotrichum* isolates and *Pestalotiopsis* isolates were distinguished using DGGE analysis. This analysis clearly separated the Colletotrichum isolates from Pestalotiopsis isolates based on their GC content and hence the banding patthern. Fagbola and Abang (2004) distinguished *Colletotrichumcircinans* and *C. coccodes* isolates based on DGGE analysis of their amplified fragments in spite of the failure of previous attempts at genetic differentiation of the two species based on RFLP analysis of the rDNA ITS region.

In the present study *C. gloeosporioides* and *Pestalotiopsis* isolates were evaluated for their genetic variability using RAPD markers. The random primers OPA-1, OPA-4, OPD-6 and OPD-12 were used. Out of these 4 primers used, OPA-1 produced reproducible banding pattern. Xiao *et al* (2004) used random amplified polymorphic DNA (RAPD) markers to determine genetic relationships among isolates recovered from noncultivated hosts and diseased strawberry plants. Phylogenetic analysis using RAPD marker data divided isolates of *C. gloeosporioides* from noncultivated hosts into two separate clusters. One cluster contained 50 out of the 52 isolates and a second cluster contained 2 isolates that were homothallic in culture. Isolates from strawberry were interspersed within the cluster containing the 50 isolates that were recovered from the noncultivated hosts. The results were not inconsistent with the hypothesis that *C. gloeosporioides* isolates obtained from strawberry and non-cultivated hosts adjacent to strawberry fields are from the same population. Gupta *et al* (2010) collected twenty-five isolates of *Colletotrichum gloeosporioides* causing mango anthracnose from different agroclimatic zones of India. These isolates were evaluated for their pathogenic variability on mango seedlings and genetic characterization using random amplified polymorphic DNA (RAPD molecular techniques). The random primers OPA-1, 3, 5, 9, 11, 15, 16 and 18

were used and the twenty five isolates were grouped into two. Out of eight primers in RAPD, OPA-1, 3 and 18 were able to produce reproducible banding pattern. Each of these primers generated a short spectrum of amplicons, located between 661 and 2291-bp markers, indicative of genetic polymorphism. Dendogram revealed more than 75% level of similarity. The data suggest that RAPD may be of value by virtue of its rapidity, efficiency and reproducibility in generating genetic fingerprints of *C. gloeosporioides* isolates. Joshi *et al* (2009) studied the genetic diversity of 22 *Pestalotiopsis*isolates causing grey blight disease of tea (*Camellia sinensis*) using two molecular marker system, RAPD and ISSR. The UPGMA clustering of both the methods were comparable and the results indicated that, within the southern India, the diversity of Pestalotiopsis was high both morphologically and genetically.

The next phase of our study was to check for antagonistic activity of selected PGPR and PGPF against the fungal pathogens of som plants. Two different *Trichoderma* isolate – *T. harzianum* and *T. asperellum* was used for in vitro antagonistic study where T. harzianum isolate RHS/480 showed better antagonistic effect against both the pathogens *C. gloeosporioides* and *P. disseminata* than *T. asperellum*. Hence further study was carried out using *T. harzianum* isolate. Rabha et al (2015) studied in vitro antagonistic activity of *T. harzianum* isolate PBR1, isolated from native rhizosphere of som plant against two important foliar fungal pathogen *Pestalotiopsis disseminata* and *Phylostictapersea*. Sunar *et a l*(2014a) tested three species of *Trichoderma* viz. *T. harzianum, T. erinaceum*and *T. asperellum* for their *in vitro* effects against *S. rolfsii*and theinhibition percentage was up to 75 percent in *T. harzianum* which was taken up forfurther *in vivo* assay and was effective in suppressing sclerotial rot of *Glycine max* causedby *S. rolfsii*. Similarly two different PGPR viz. *Bacillus pumilus* and *Bacillus altitudinus* was also taken into consideration for checking their *in vitro* antagonistic activity against the fungal pathogens. *B.pumilus* showed better activity against *C. gloeosporioides* and *B. altitudinus* showed better antagonism towards *Pestalotiopsis* sp. Hence these PGPR were further utilised for their in vivo assay against their respective pathogens.

Changes in levels of different defense related enzymes, viz. Phenylalanine ammonia lyase (PAL), Peroxidase (POX), Chitinase (CHT) and β-1,3Glucanse (GLU) following infection with *Colletotrichumgloeosporioides*was also studied during this investigation. Presence of new peroxidase isozyme in infected leaf samples was also recorded (Chakraborty *et al.,* 2016b).Correlation of this result was also made with the

study undertaken by Parihar*et al* (2012) where it was seen that biochemical analysis of genotypes of *Brassica juncea*infected with *Alternaria* blight revealed an increase in PAL, PPO and peroxidase activity. Singh *etal* (2014) reported that preformed phenolic compounds as well as Peroxidase enzyme play important role in resistance of Chili against *Colletotrichum capsici.* Analysis of peroxidase isozymes by polyacrylamide gel electrophoresis showed four isozymes in healthy tea leaf samples and five in tea leaves infected with *Exobasidium vexans*. They suggested that the appearance of new bands following infection can be correlated with the induction of the catalytic activity of more isozymes, leading also to an overall increase in peroxidase activity (Chakraborty *et al.*, 2002)

In the present study it was observed that total as well as ortho-dihyroxy phenol content increased in infected plants, more in plants with less infection. Infection by *Venturiainequalis* in apple caused an accumulation of phenolic compounds wherein Folin-Ciocalteu values increased by 1.4 to 2.4 fold (Petkovsek *et al*., 2008). Taware *et al* (2010) studied that there was significant increase in total phenolic content of grape leaves due to foliar powdery mildew infection. These results are in accordance with the result obtained in the present study.

Thin layer chromatography has been used by various workers to study the phenol profile of different plants like tea (Chakraborty and Saha, 1994), som (Neog *et al.*, 2011), different medicinal plants (Maobe *et al*., 2012). Studies on secondary metabolites of som plant by Neog *et al* (2011) revealed the presence of four major phenolic acids – Chlorogenic acid, Catechol, Morin and Gallic Acid. In the present study HPLC analysis revealed the presence of Resorcinol, catechol and chlorogenic acid in both healthy and infected leaves (*Colletotrichum gloeosporioides*) but the height of these peaks increased in infected samples. In addition presence of two new peaks in infected sample could be identified as ferulic acid and salicylic acid. Results revealed that the pathogen triggered the production of resorcinol, catechol, chlorogenic acid, ferulic acid and salicylic acid in the muga host plant as biochemical defense strategy (Chakraborty *et al*., 2016 b). Presence of chlorogenic acid as part of defense system has been studied by different workers in different crops, such as Potato tubers (Malamberg and Theander, 2011), apples (Petkovsek 2003), coffee (Rodrigues 2011) and tomato (Lopez-Gresa*et al.*, 2011). Presence of salicylic acid and ferulic acid in infected leaves and not in healthy leaves indicate the role of this phenolic acid in defense against pathogen. When biochemical charecterization of

maize plants infected with *Drechslera dactylidis* was done, it was found that salicylic acid increased 2-fold in infected leaf samples (Ghany 2012).

Activation of defense response in som plants was observed after application of bioinoculants against *C.gloeosporioides* and *P. disseminata*. The association of AMF with the Som plants was studied and their diversity was assessed in eight morphotypes. Among these, *Glomus* sp, *Scutellospora* sp, *Acaulospora* sp and *Gigaspora* sp were foundassociated with the different morphotypes of Som plants, wherein *Glomus* sp was predominant. Growth promotion in Som plants following successful root colonization with AMF was noted in terms of increase in height, no of leaves and no of branches. Two main defense enzymes in plants, Phenylalanine Ammonia Lyase (PAL) and Peroxidase (POX) were found higher in AMF inoculatedplants in comparison to control plants indicating induced resistance in Som plants(Chakraborty *et al*., 2013). Application of *B. pumilus* and AMF, singly or jointly increased growth of som morphotypes in pot as well as in field condition, but better result was obtained when both were co-inoculated. Enhanced accumulation of total protein, phenolics as well as increased activities of major defense enzymes peroxidase, phenylalanine ammonia lyase, chitinase and β1,3 glucanase were also observed following the above mentioned treatments in som plants. Dual application of AMF and PGPR also suppressed the disease incidence of leaf blight of som plants following artificial inoculation of treated plants with *C. gloeosporioides*(Chakraborty *et al*; 2014). A significant improvement in growth measured in terms of increase in shoot length, number of leaves and branches was observed when one year old som saplings were bacterized with a strain of *Pseudomonas* under pot conditions (Rabha *et al.*, 2014). Increased activity of chitinase, β-1, 3-glucanase and peroxidase were alsodetermined in tea plants following treatments with Josh - a bioformulation of AMF (Chakraborty *et al.,* 2007).

Growth and yield of various crops have been reported to increase following application of Vermicompost along with other bioinoculants such as tomato (Bhattacharjee*et al*., 2015), Chickpea (Sahni *et al*., 2008), guava (Pathak *et al*., 2013). In accordance with these observations, in the present study it was observed that application of Vermicompost, PGPR and AMF, alone and in combination increased growth of eight different morphotypes of som plants. Enhancement of total phenol as well as defense related enzymes was seen in dual and triple treatments irrespective of the morphotypes. Among the various treatments a consistent decrease in disease

incidence of grey blight after artificial inoculation of P. disseminata spores was observed in plants treated with PGPR followed by Vermi + PGPR treatment when compared to the untreated inoculated plants. It was seen that disease progression in treated plants were very slow when compared with untreated plants (Acharya *et al*., 2015b).

Effect of combined application of three different bioinoculants - PGPR, AMF and PGPF, on growth as well as suppression of leaf blight disease of som plant was also studied in this investigation. It was recorded that under nursery condition, growth enhancement was noticeable after 60 days of combined treatment of all bioinoculants. Two morphotypes S5 and S6 were shown highest growth and was transferred to field where the soil was amended with bioformulation of PGPR, PGPF and mass multiplied AMF. Total protein, chlorophyll content as well as total and ortho-phenol content of treated plants was increased than control plants. Unni*et al*(2008) found that five different PGPRs helped improve the growth of the som plant by increasing chlorophyll content, free amino acid, total protein, reducing sugar, carbohydrate and dry weight.Balamurugan*et al* (2013) explained in his studies with *Camellia sinensis* that during the first six months after application of inorganic fertilizers (IOF) and bioinoculants (BF), the results revealed that recommended dosage of the fertilizers provided significantly higher values of biometric characteristics in terms of stem height, no. of leaves, biomass production in tea seedlings. Results of chlorophyll content of the leaves sampled after six months revealed that application of 100% IOF+BF had shown higher total chlorophyll content followed by 50% IOF+BF and 100% IOF alone. But after 12 months, chlorophyll content was higher after application of 100% IOF+BF followed by IOF alone.

Disease symptoms were established in the plants sprayed with spore suspension of *C. gloeosporioides* well after 96 hrs of inoculation. It was noted that symptoms appeared in untreated inoculated leaves much faster than in treated inoculated plants. Results of Percent disease index correlate with the results obtained from the analysis of defense enzymes where the enzyme activity of PAL, POX, CHT and GLU was much higher in treated inoculated leaves indicating their increased defense against the pathogen. But the healthy leaves showed more levels of enzymes suggesting that the inoculated leaves are prone to disease development where the enzyme levels were less. Application of *G. mosseae*and *T. hamatum*singly or jointly suppressed root rot of mandarin caused by *F. solani*. Defense enzymes, chitinase, glucanase and peroxidase

showed enhanced activitiesduring disease suppression (Allay and Chakraborty, 2010). Chakraborty *et al*., 2016 reported that dual application of *Bacillus pumilus* and *Rhizophagusfasciculatus* caused induction of resistance in *Camellia sinensis* against *Sclerotiumrolfsii*. It is now clear that microbes in small consortia enhance the defense signalling cascades leading to enhance transcriptional activation of several metabolic pathways (Sarma*et al*., 2015).

In the next phase of our study, induction of defense enzymes mainly chitinase was studied using fluorescent antibody staining technique. Leaves of som plants treated with PGPR and roots treated with AMF and *T. harzianum* were reacted with Pab of chitinase followed by labelling with FITC. Strong bright apple green fluorescence was observed in the epidermal and homogenously in mesophyll tissues inleaves and homogenously in cortical cells and epidermal cells in roots. Enhancement ofchitinase was revealed in both leaves and roots following induction.Chakraborty *et al* (2009) studied the expression of chitinase in leaves of treated tea plants following induction with salicylic acid using immunofluorescent techniques.

Simultaneously immunogold localization of defense enzyme chitinase in leaves of som plants treated with PGPR as well as roots treated with AMF and PGPF was studied through Transmission Electron Microscopy. Heavy deposition of gold particles was observed in cytoplasm of leaf section confirming the expression of chitinase following treatment with bioinoculants. Yedida*et al*(1999), observed that application of *Trichoderma* to rhizosphere of cucumber seedlings initiated a series of morphological and biochemicalchanges. Gold labeling was done with β-1,4-exoglucanase and β-1,3-glucanase whereintense gold labeling was observed over both the cell walls and wall appositions. The deposition of the chitinase following treatment with salicylic acid was found predominantly in cellular compartments of ultrathin sections of tea leaves. Gold labelling in the sections showed a high amount of labelling in chloroplasts and hostcytoplasm and lesser amount in vacuoles, mitochondria and walls(Chakraborty *et al*., 2009). This suggests the potential of salicylic acid for protection of tea against foliar pathogens.

It is evident from the results that observed growth promotion and enhanced resistance to pathogen due to joint application of bioinoculants (AMF, PGPF and PGPR) can be attributed to activation of defense responses in som plants such as increased accumulaton of phenolics and defense enzyme activities.