Chapter 3
MATERIALS AND METHOD

3.1. Plant Material

3.1.1. Collection

Eight different morphotypes of *Persea bombycina* (viz. S1, S2, S3, S4, S5, S6, S7 and S8) were collected from Central Muga Eri Research and Training Institute (CMER&TI), Jorhat, Assam. These different morphotypes of som are classified based on their leaf shape. Commonly they are called as Ampotia (S1 and S2), Naharpotia (S3), Jampotia (S4), Belpotia (S5), Kothalpotia (S6) etc.

3.1.2. Maintenance in glass house

The eight different morphotypes of som plant were maintained in glass house of Immuno-phytopathology Laboratory, Department of Botany, University of North Bengal. Minimum 15 plants of each morphotypes were maintained in earthenware pots (12" dia) (Figure 1)

3.1.3. Maintenance in field condition

One year old saplings of all eight morphotypes were transferred to Experimental field in Padmaja Park of University of North Bengal where all suitable management practices were taken into consideration for proper growth of the plants. Two different morphotypes (S5 and S6) that showed the best growth activity under nursery and glass house and nursery condition were transferred to experimental field of immune-phytopathology Laboratory, NBU for close monitoring and further experimentation. (Figure 2)

Figure1: Maintenance of *Persea bombycina* morphotypes in glass house condition

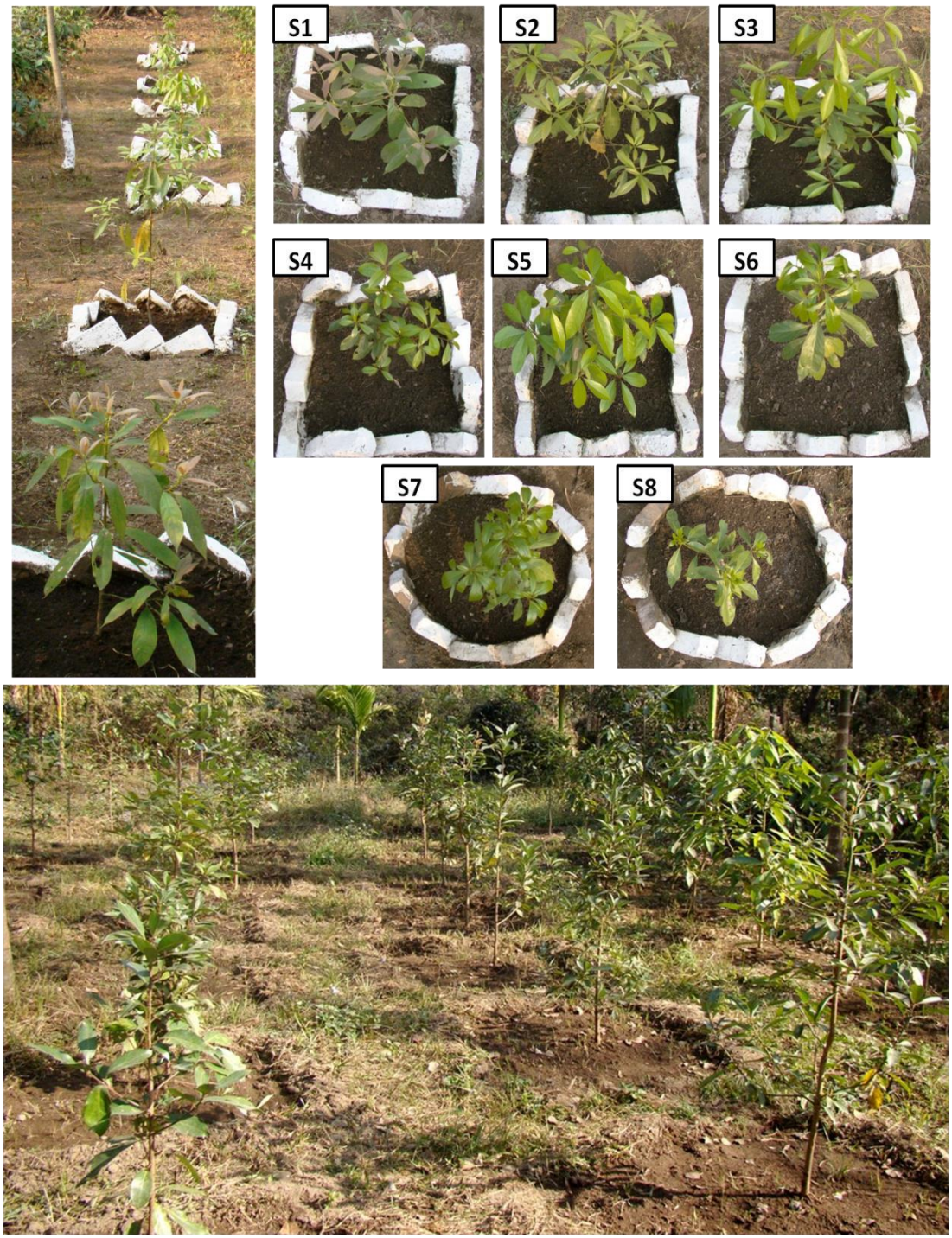

Figure2: Maintenance of *Persea bombycina* morphotypes in field condition

3.2. Fungal Culture

3.2.1. Isolation and maintenance

The fungal pathogens which they are present in deep seated tissue of infected leaves were isolated by culturing pieces of internal tissues. Infected tissues were thoroughly washed in sterile water and then swabbed with cotton wool dipped into 80% ethanol, followed by exposure to an alcohol flame for a few seconds. The outer layer of tissues was quickly removed by a flame sterilized scalpel. Small pieces from the central core of tissue in the area of the advancing margin of infection were removed by a sterilized scalpel and sterilized by dipping into 90% alcohol then flamed for a few seconds. The sterilized tissues were transferred to potato dextrose agar in Petri dishes and incubated at 28ºC for 1 week. The fungal mycelium grown was transferred to PDA slants and kept for further identification.

3.2.2. Morphological and microscopic observation

The isolated fungi were allowed to grow in Petriplates (7cm) containing sterile PDA medium for 7 days, then nature of mycelia growth, rate of growth and time of sporulation were observed. For identification, spore suspension was prepared from individual culture. Drops of spore suspensions were placed on clean, grease free glass slides, mounted with lacto phenol- cotton blue, covered with cover slip and sealed with wax. The slides were then observed under the microscope following which spore characteristics were determined and size of spores measured.

3.2.3. Completion of Koch's Postulate

Fresh young som leaves were collected from experimental field of Immuno-Phytopathology lab and inoculated with conidial suspension of the isolated fungal pathogen following detached leaf inoculation technique. After 96h of inoculation, the infected som leaves were washed thoroughly, cut into small pieces, disinfected with 0.1% $HgCl_2$ solution for 3-5 min washed several times with sterile distilled water and transferred aseptically into Potato Dextrose Agar (PDA) slants. These isolates were examined 15 days of inoculation at 30°C and identity of the organism was confirmed by comparing with the stock culture.

3.2.4. Assessment of mycelia growth

Mycelial growth of the fungal cultures was assessed in both solid media and liquid media to study and evaluate their cultural characteristics.

3.2.4.1. Solid Media

To assess the growth of fungal culture in solid media, the fungus was first grown on petri dishes, each containing 20ml of PDA followed by incubation for 7 days at 30ºC. Agar blocks (6mm diameter) containing the mycelium was cut with sterile cork borer from the actively growing region of mycelial mat and transferred to each Petri dish containing 20ml of different sterilized solid media. The colony diameter was studied at regular interval of time. The media were as follows:

A. Potato Dextrose Agar (PDA)

Peeled potato - 40.00g, Dextrose – 2.00g, Agar - 2.00g, Distilled water - 100ml

B. Richard's Medium (RMA)

KNO_3 - 1.00g, KH_2PO_4 - 50g, $MgSO_4.7H_2O$ - 0.25g, $FeCl_3$ - 0.002g, Sucrose - 3.00g, Agar- 2.00g, Distilled water – 100ml

C. Oat Meal Agar (OMA)

Oat meal – 3.00g, Agar – 2.00g, Distilled water – 100ml

3.2.4.2. Liquid Media

To assess the mycelial growth in liquid media the fungus was first grown on petriplates, each containing 20ml of PDA and incubated for 5-8 days at 28ºC. The mycelial block (5mm) from the actively growing region of the fungus in the petriplate was cut with sterilized cork borer and transferred to Erlenmeyer flask (250ml) containing 50 ml of sterilized Potato dextrose broth (PDB) and Richards medium and incubated for 6 - 8 days with constant stirring at room temperature. After incubation the mycelia were harvested through muslin cloth, collected in aluminium foil cup of known weight and dried at 60ºC for 96 h, cooled in desiccators and weighed.

3.3. Soluble proteins

3.3.1. Extraction of soluble protein

3.3.1.1. Fungal mycelia

Mycelial protein was prepared following the protocol as outlined by Chakraborty and Saha (1994). The fungal mycelia were grown in 250 ml Erlenmeyer flask each containing 50 ml of potato dextrose broth (PDB) and incubated for 10 days at 30+ 1ºC. for extraction of antigen, mycelial mats were harvested washed with 0.2% NaCl and rewashed with sterile distilled water. Washed mycelia were crushed with sea sand using a chilled mortor and pestle and homogenized with cold 0.05m sodium phosphate buffer (PH-7.2) supplemented with 0.85% NaCl, 10mM sodium metabisulphite, PVPP (Polyvinyl pyrollidine Phosphate) and 0.5mM magnesium

chloride in ice bath. The homogenated mixture was kept for 2h or overnight at 4ºC and then centrifuged at 10.000rpm for 30 min, at 4ºC to eliminate cell debris. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring in ice bath and kept overnight at 4ºC. After this period, the mixture was centrifuged (10,000rpm) for 30 minute at 4º C, the precipitate was dissolved in the same buffer (pH 7.2). The preparation was dialysed for 72h through cellulose tubing (sigma chemical co., USA) against 1L of 0.05 M sodium phosphate buffer (pH 7.2) with six changes. The dialysate was stored at -20ºC and used as antigen from the preparation of antiserum and other experiment.

3.3.1.2. Leaf

Soluble protein was extracted from som leaves following the method of Chakraborty *et al*., (1995). Leaf tissues were frozen in liquid nitrogen and ground in 0.05mM Sodium phosphate buffer (pH 7.2) containing 10mM $Na_2S_2O_5$, 0.5mM $MgCl_2$and 2mM PVP was added during crushing and centrifuged at 4ºC for 20 min at 12000rpm. The supernatant was used as crude protein extract.

3.3.2. Estimation of soluble protein content

Soluble proteins were estimated following the method as described by Lowry *et al.*, (1951). To 1ml of protein sample 5ml of alkaline reagent (1ml of 1% CuSO4 and1ml of 2% sodium potassium tartarate, added to 100ml of 2% $Na_2 CO_3$ in 0.1 NaOH) wasadded. This was incubated for 15 min at room temperature and then 0.5ml of 1N FolinCiocalteau reagent was added and again incubated for further 15 min following whichoptical density was measured at 720 nm. Quantity of protein was estimated from thestandard curve made with bovine serum albumin (BSA).

3.4. SDS-PAGE analysis of soluble proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for detailed analysis of protein profile following the method of (Laemmli, 1970). For the preparation of gel the following stock solution were prepared.

3.4.1. Preparation of stock solution

Following stock solution were prepared

A. Acrylamide and N'N' – methylene bis acrylamide

Stock solution containing 29% acrylamide and 1% bis-acrylamide was prepared in warm water, as both of them are slowly dominated to acrylic and bis acrylic acid by alkali and light. The pH of the solution was kept below 7.0 and the stock solution was

then filtered through Whatman No. 1 filter paper and kept in brown bottle, stored at 4ºC and used within one month.

B. Sodium Dodecyl Sulphate (SDS)

A 10% stock solution of SDS was prepared in warm water and stored at room temperature.

C. Tris Buffer

i) 1.5M Tris buffer was prepared for resolving gel. The pH of the buffer was adjusted to 8.8 with concentrated HCl and stored at 4ºC for further use.

ii) 1.0 M Tris buffer was prepared for use in the stacking and loading buffer. The pH of this buffer was adjusted to 6.8 with conc. HCl and stored at 4ºC for use.

D. Ammonium Persulphate (APS)

Fresh 10% APS solution was prepared with distilled water each time before use.

E. Tris- Glycine electrophoresis buffer

Tris running buffer consists of 25mM Tris base, 250mM Glycine (pH 8.3) and 0.1% SDS. A 1X solution was made by dissolving 3.02 g Tris base, 18.8 g Glycine and 10 ml of 10% SDS in 1L distilled water.

F. SDS gel loading buffer

This buffer contains 50 mM Tris –HCl (pH 6.8), 10 mM ß- mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol. A 1x solution was prepared by dissolving 0.5ml of 1M Tris buffer (pH 6.8), 0.5ml of 14.4 M ß- mercaptoethanol, 2 ml of 10% SDS, 10 mg bromophenol blue, 1ml glycerol in 6.8 ml of distilled water.

3.4.2. Preparation of gel

Mini slab gel (plate size 8cm x10cm) was prepared for analysis of proteins patterns through SDS-PAGE. For gel preparation, two glass plates (8 cmx10 cm) were washed with dehydrated alcohol and dried to remove any traces of grease. Then 1.5 mm thick spacers were placed between the glass plates at the two edges and the three sides of the glass plates were sealed with gel sealing tape or wax, clipped tightly to prevent any leakage and kept in the gel casting unit. Resolving and stacking gels were prepared by mixing compounds in the following order and poured by pipette leaving sufficient space for comb in the stacking gel (comb +1cm). After pouring the resolving gel solution, it was immediately over layered with isobutanol and kept for polymerization for 1h. After polymerization of the resolving gel was complete,

overlay was poured off and washed with water to remove any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and the comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30-45 minutes. After polymerization of the stacking gel the comb was removed and the wells were washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris – Glycine buffer was added sufficiently in both upper and lower reservoir. Any bubble trapped at the bottom of the gel, was removed carefully with a bent syringe.

Name of the compound	10% Resolving Gel (ml)	5% Stacking Gel (ml)
Distilled Water	2.85	2.10
30% Acrylamide	2.55	0.50
Tris*	1.95	0.38
10%SDS	0.075	0.030
10%APS	0.075	0.030
TEMED**	0.003	0.003

*For 1.5 M tris pH 8.8 in resolving gel and for 1M Tris pH 6.8 in stacking gel

** N,N,N‘,N‘ –Tetramethyl ethylene diamine

3.4.3. Sample preparation

Sample (50µl) was prepared by mixing the protein extract (35 µl) with 1xSDS gel loading buffer (16 µl) in cyclomixer. All the samples were floated in a boiling water bath for 30 minutes to denature the proteins samples. After boiling, the sample was loaded in a predetermined order into the bottom of the well with T-100 micropipette syringe. Along with the protein samples, a marker protein consisting of a mixture of six proteins ranging from high to low molecular mass (Phosphorylase b-97,4000: Biovine Serum Albumin - 68,000; Albumin -43,000; Carbolic Anhydrase -29.000; Soybean Trypsin inhibitor- 20,000; Lysozyme - 14,300) was treated as the other samples and loaded in separate well.

3.4.4. Electrophoresis

Electrophoresis was performed at 18mA current for a period of two to three hours or until the dye reached the bottom of the gel.

3.4.5. Fixing and staining

After completion of electrophoresis, the gel was removed carefully from the glass plates and the stacking gel was cut off from the resolving gel and finally fixed in

glacial acetic acid: methanol: water (10:20:70) for overnight. The staining solution was prepared by dissolving 250mg of Coomassie brilliant blue (Sigma R 250) in 45 ml of methanol. When the stain was completely dissolved, 45ml of water and 10ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No.1 filter paper. The gel was removed from the fixer and stained in this stain solution for 4 h at 37 ºC with constant shaking at low speed. After staining the gel was finally destained with destaining solution containing methanol, water and acetic acid (4.5: 4.5:1) at 40 ºC with constant shaking until the background become clear.

3.5. Preparation of antigen

3.5.1. Fungal antigen

Mycelial protein was prepared following the method as outlined by (Chakarborty and Saha 1994). Mycelia mats were harvested from 7-10 days old culture and washed with 0.2% NaCl then again rewashed with sterile distilled water. Washed mycelia were crushed with sea sand using a chilled mortar and pestle and homogenized with cold 0.05 M sodium phosphate buffer (pH 7.2) supplemented with 0.85% NaCl, 10 mM sodium metabisulphite and 0.5 mM $MgCl_2$ in ice bath. The homogenate mixture was kept for 2h or overnight at 4 ºC and then centrifuged at 10,000rpm for 30 min at 4 ºC to eliminate cell debris. The supernatant was collected and stored in -20 ºC and used as antigen for the preparation of antiserum.

3.5.2. Leaf Antigen

3.5.2.1. Healthy leaf

Antigens from healthy leaves were prepared following the method of Chakraborty and Saha (1994). Fresh, young healthy leaves were collected from the experimental garden and kept at 4°C. Then the leaves were weighed and crushed in mortar and pestle with 0.05M Sodium phosphate buffer supplemented with 10mM Sodium metabisulphite, 2mM PVPP 10,000 (soluble) and 0.5mM magnesium chloride (pH 7.2). At the time of crushing with sea-sand insoluble PVPP of equal weight was used. The leaf slurry was strained through a muslin cloth and then centrifuged (15,000g) for 30 min at 4°C. the supernatant was used as healthy leaf antigen and was kept at -20°C until required.

3.5.2.2. Artificially inoculated leaf

Antigen from *C. gloeosporioides* and *P. disseminata* inoculated leaves were extracted following the method of Alba & DeVay (1985) with modification. Fresh, young leaves were collected from experimental garden and kept in plastic trays as described in detached leaf inoculation technique. Leaves were inoculated with conidial suspension of both the pathogens separately. Control sets were prepared by mounting the leaves with drops of sterile distilled water. Antigens were prepared from inoculated leaves as well as control leaves as described earlier. The prepared antigens were stored at -20°C until further experimental purposes.

3.5.2.3. Naturally infected leaf

For the extraction of naturally infected leaf antigens, the infected leaves were collected from the experimental garden and kept at 4°C. Then the infected portion of leaf was cut into small pieces, weighed and antigens were prepared as before.

3.5.3 AMF antigen

AMF spores were isolated from rhizosphere soil of som by wet sieving and decanting method (Gerdemann and Nicolson, 1963). With the help of a dissecting microscope parasitized spores, plant debris etc were separated and clean AMF spores were isolated. These spores were sonicated with 0.1% normal saline under the frequency range of 70-75 mhz as impulse. The supernatant was used as antigen source.

3.6. Serology

3.6.1. Rabbits and their maintenance

Polyclonal antibodies were prepared against fungal and bacterial antigens in New Zealand white male rabbits of approximately 2kg of body weight. Before immunization, the body weights of rabbits were recorded and observed for at least one week inside the cages. Rabbits were maintained in Antisera reserves for plant pathogens, Immuno-Phytopathology Laboratory, Department of Botany, NBU. They were regularly fed with green grass, soaked gram, green vegetables and carrots etc. twice a day. After each bleeding they were given saline water for three consecutive days and kept in proper hygienic conditions.

3.6.2. Immunization

Before immunization, normal sera were collected from each rabbits. For developing antisera , intramuscular injections of 1ml antigen(protein extracted) mixed with 1ml of Freund‘s complete adjuvant (Genei) were given into each rabbit 7 days after pre-immunization bleeding and repeating the doses at 7 days intervals for consecutive

week followed by Freund‘s incomplete adjuvant (Genei) at 7 days intervals upto 12-14 consecutive weeks as required. Method of (Alba and Devay, 1985) and (Chakraborty and Saha, 1994) were followed for immunization.

3.6.3. Bleeding

Bleeding was performed by marginal ear vein puncture, three days after the first six injections, and then every fourth injection. In order to handle the rabbits during bleeding, they were placed on their back on a wooden bored fixed at an angle of 60º, and held the rabbits tight so that it could not move during the bleeding. The hairs from the upper side of the ear was removed with the help of a razor and disinfected with alcohol. The ear vein was irritated by the application of xylene and an incision was made with the help of a sharp sterile blade and 5 -10 ml of blood samples were collected in sterile graduated glass tube. The blood samples were incubated at 37ºC for 1hr for clotting. After clotting; the colt was loosened with a sterile needle. Finally, the serum was classified by centrifugation. (2000g for 10 minute at room temperature) and distributed in 1 ml vials and stored at -20ºC as crude antisera. The serum was used for double diffusion analysis, dot blots analysis and Enzyme Linked Immunosorbent Assay (ELISA).

3.6.4. Purification of IgG

3.6.4.1. Precipitation

IgG was purified as described by (Clausen, 1988). Crude antiserum (2ml) was diluted with two volume of distilled water and an equal volume of 4M (NH_4SO_4) ammonium sulphate was taken and pH adjusted to 6.8, stirring the mixture for 16h at 20º C in magnetic stirrer. The precipitate thus formed was collected by centrifugation at 12,000 rpm for 1h at 22 º C for 1 h. Supernatant was discarded and pellet was used for further steps.

3.6.4.2. Column preparation

Eight gram of DEAE cellulose (Sigma Co. USA) was suspended in distilled water for overnight. The water was poured off and the DEAE cellulose was suspended in 0.005M phosphate buffer (pH 8.0) and the washing was repeated for 5 times. The gel was then suspended in 0.02 M phosphate buffer, (pH 8.0) and was transferred to a column (2.6 cm in diameter and 30cm height) and allowed to settle for 2h. After the column material had settled 25ml of buffer (0.02M sodium phosphate, pH 8.0) washing was given to the column material.

3.6.4.3. Fraction collection

At the top of the column, 2ml of ammonium sulphate precipitate was applied and the elution was performed at a constant pH and a molarity continuously changing from 0.02 M to 0.03 M. the initial elution buffer (1) was 0.02 M sodium phosphate (pH 8.0). The buffer was applied in the flask on which rubber connection from its bottom was supplying column. Another connection above the surface of buffer (1) was connected to another flask with buffer (2). The buffer (2) had also connection to the open air. During the draining of buffer (1) to column buffer (2) was soaked into buffer (1) thereby producing a continuous raise in morality. Ultimately, 40 fractions each of 5ml were collected and the optical density values were recorded at 280nm using UV-Vis spectrophotometer (DIGISPEC-200GL).

3.7.Immunological assays

3.7.1. Agar gel double diffusion

3.7.1.1. Preparation of agarose slides

The glass slides (6cm x 6cm) were degreased using ethanol 90%v/v: diethyl ether (1:1v/v) and ether, then dried in hot air oven. After drying the plates were sterilized inside the petriplate each containing one plate. Conical flask with Tris-Barbiturate buffer (pH 8.6) is placed in boiling water bath. Agar/ agarose (0.9%) was boiled over water bath to dissolve the agar at 90 º C for next 15 min. Then pinch of 0.1% (w/v) sodium azide was added and mixed well. For the preparation of agarose gel, the molten agarose is poured (6 to 10 ml) on the grease free sterilized slide with the help of a sterile pipette in laminar air flow chamber and allow it to solidify, after solidification cut 3-7 wells (6mm diameter) with sterilized cork borer distance of 1.5 to 2cm away from central well and 2.0 to 2.5 cm from well to well.

3.7.1.2. Diffusion

Agar gel double diffusion tests were carried out using antigen and antiserum following the method of Ouchterlony (1967). Antigen plus undiluted antisera appropriately diluted were poured into wells with micropipette (50µl/well) antisera in middle. Slides were kept in moist chamber at 25ºC for 72h. Precipitation reaction was observed in the agar gel only in cases where common antigen was present.

3.7.1.3. Washing, staining and drying of slides

After immunodiffusion, the slides were initially washed with sterile distilled water and then with aqueous NaCl solution (0.9% NaCl and 1% NaN_3) for 72 h with 6

hourly changes to remove unreacted antigens and antisera widely dispersed in the agarose gel. Then the slides were stained with Coomassie brilliant blue (R250, Sigma: 0.25g Coomassie blue, 45ml methanol, 45ml distilled water and 10ml glacial acetic acid) for 10 min at room temperature. After staining, the slides were washed in destaining solution (methanol: distilled water: acetic acid in 45:45:10 ratios) with changes until background become clear. Finally slides were washed with distilled water and dried in hot air oven for 3 h at 50º C.

3.7.2. Plate trapped antigen coated (PTA) - ELISA

Plate trapped antigen coated (PTA)-ELISA was performed following the method as described by (Chakraborty *et al.*., 1995) with modifications. Antigen were diluted with coating buffer and the antigens were loaded (200µl per well) in ELISA plate (Coaster EIA/RIA, strip plate, USA) arranged in12 rows in a (Cassette) ELISA plate. After loading, the plate was incubated at 25 ºC for 4 h. then the plate was washed four times under running tap water and twice with PBS-Tween and each time shaken well to dry. Subsequently, 200 µl blocking reagent was added to each well for blocking the unbound sites and plate was incubated at 25 ºC for 1h. After incubation, the plate was washed as mentioned earlier. Purified polyspecific IgG was diluted in antisera dilution buffer and loaded (200 µl per well) and incubated at 4ºC overnight. After a further washing, antirabbit IgG goat antiserum labeled with alkaline phosphatase diluted 10000 times in PBS, was added to each well (100 µl per well) and incubated at 37 ºC for 2 h. The plate was washed, dried and loaded with 200 µl of p-Nitrophenyl Phosphate substrate in each well and kept in dark for 1 h. color development was stopped by adding 50 µg per well of 3 N NaOH solution and the absorbance was determined in an Multiscan Ex (Thermo Electron) ELISA Reader (Multiskan, Thermo Labsystems) at 405nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.7.3. Dot immunobinding assay

Dot blot was performed following the method suggested by Lange *et al.*,(1989) with modifications. Following buffers were used for dot immunobinding assay.

A. Carbonate –bicarbonate (0.05 M, pH 9.6) coating buffer.

B. Tris buffer saline (10mM pH 7.4) with 0.9% NaCl and 0.5% Tween 20 for washing.

C. Blocking solutions 10% (w/v) skim milk powder (casein hydrolysate, SLR) in TBST (0.05 M Tris-HCl, 0.5 M NaCl) 5% v/v Tween 20 , pH 10.3.

D. Alkaline phosphatase buffer (100 mM tris HCl, 100 mM NaCl, 5mM $MgCl_2$)

Nitrocellulose membrane (Millipore, 7cm x10cm, Lot No. H5SMO 5255, pore size 0.45µm, Millipore corporation, Bedford) was first cut carefully into the required size and fix between the template with filter paper at the bottom. 0.5M carbonate-bicarbonate buffer (pH 9.6), 4µl, was loaded in each well and allowed to dry for 30 min at room temperature. Antigen (5µl) was loaded on to NCM and allowed to dry for 30 min at room temperature. Template was removed and blocking of NCM was done with 19% non fat dry milk (casein hydrolysate, SRL) prepared in TBST for 30-60 minutes on a shaker. Respective polyclonal antibody (IgG 1:500) prepared against that antigen was added directly in the blocking solution and further incubated at 4 ºC for overnight. The membrane was then washed gently in running tap water for three min, thrice followed by washing in TBST (pH 7.4), (Wakeham and White, 1996). The membrane was then incubated in alkaline phosphatase conjugated goat antirabbit IgG (diluted 1:10,000 in alkaline phosphatase) for 2h at 37ºC. The membrane was washed as before. 10 ml of NBT/BCIP substrate (Genei) was added next and color development was stopped by washing the NCM with distilled water and color development was categorized with the intensity of dots.

3.7.4. Western blot analysis

Protein samples were electrophoresed on 10% SDS-PAGE gels as suggested by Laemmli (1970) and electro transferred to NCM using semi-dry Trans-blot unit (BioRad) and probed with PAbs of the pathogen (*C. gloeosporioides* and *P. disseminata*) following the method of Wakeham and White (1996). Hybridization was done using alkaline phosphatase conjugate and 5-bromo-4-choloro-3-indolylphosphate (NBT-BCIP) as substrate. Immunoreactivity of the proteins was visualized as violet coloured bands on the NCM

3.7.5. Fluorescence antibody staining and microscopy

Indirect fluorescence staining of fungal mycelia, cross- section of mandarin roots and leaves were done using FITC labeled goat antirabbit IgG following the method of (Chakraborty and Saha, 1994). Both FITC and RITC were done to locate AMF spores in soil and observe root colonization and cellular location of AMF which was mass multiplied in mandarin roots following colonization with AMF.

3.7.5.1. Fungal mycelia

Fungal mycelia were grown in liquid potato dextrose medium as described earlier. After five days of inoculation young mycelia were taken out from flask and taken in

Eppendorf tube and washed with PBS (pH 7.2) by centrifugation at slow speed. Then mycelia was treated with normal sera or antisera diluted (1:50) in PBS and incubated for 1 h at room temperature. The mycelia was washed thrice with PBS- Tween (pH 7.2) as mentioned above and treated with Goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma chemicals) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 45 min at room temperature. After incubation mycelia was washed thrice in PBS and mounted in 10% glycerol. A cover slip was placed and sealed. The slides were observed and photograph under both phase contrast and UV fluorescence condition using Leica Leitz Biomed microscopy with fluorescence optics equipped in (UV) filter set 1-3.

3.7.5.2. Conidia

Fungal conidia were collected from 15 day-old culture and a suspension of this was prepared with PBS, pH 7.2. Conidial suspensions were taken in micro-centrifuge tubes and centrifuged at 3000g for 10min and the PBS supernatant was discarded. Then 200µl of diluted antisera (in PBS pH 7.2) in the ratio 1:125 was added into microcebtrifuge tube and incubated for 2h at 27°C. After incubation, the tubes were centrifuges at 3000g for 10 min and the supernatant was discarded. Then the spores were rewashed 3 times with PBS-Tween pH 7.2 by centrifugation as before and 200µl of goat antirabbit IgG conjugated FITC (diluted 1:40 in PBS) was added and the tubes were incubated in dark at 26°C for 1h. After the dark incubation excess FITC-antisera was removed by repeated washing with PBS-Tween pH 7.2 and the spores were mounted on glycerol jelly and observed under Leica Leitz Biomed microscopy with fluorescence optics equipped in (UV) filter set 1-3.

3.7.5.3. Cross section of som roots and leaves

Initially, cross section of healthy mandarin roots and leaves were cut and immersed in PBS (pH 7.2). These section were treated with normal serum or antiserum diluted (1:50) in PBS and incubated for 1 hour at room temperature. After incubation, cross sections were washed thrice with PBS- Tween (pH 7.2) for 15 minute and transferred to 40µl of diluted (1:40) goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC).The sections were incubated for 45 minutes in dark. After that sections were washed thrice with PBS- Tween as mentioned above and then mounted on a grease free slide with 10%glycerol. Fluroescence of the root and leaf sections were observed using Leica Leitz Biomed Microscope with fluorescence optics equipped with UV- filter set I-3 and photograph was taken.

3.7.5.4. AMF in som root

Roots of maize plants (4 months) and som plants (2 years old) in which AMF spores of som were mass multiplied were macerated according to Philips and Hayman method as described by with a few modification. Antigen was given in the dilution 1:50 goat antirabbit IgG after washing with PBS pH 7.2 thrice. The roots were incubated overnight in dark. The next day, the roots were again washed thrice with PBS Tween and fluorescein isothiocyanate(FITC) and rhodamine isothiocyanate (RITC) was added in the dilution 1:10 and incubated for 45 minutes in dark. The roots were again washed thrice in PBS and mounted in 10% glycerol in grease free slides. Fluroescence of the root section were observed using Leica Leitz Biomed Microscope with fluorescence optics equipped with UV- filter set I-3 and photograph was taken.

3.8. Isolation of genomic DNA

Isolation of fungal genomic DNA was done by growing the fungi for 3-4 days. Liquid nitrogen was used for crushing the cell mass for both cases.

3.8.1. Preparation of genomic DNA extraction buffer

The following buffer for DNA extraction were prepared by mixing appropriate amount of desired chemicals with distilled water and adjusted the desired pH.

DNA Extraction buffer

1M Tris-HCl pH 8.0

5M Nacl

0.5 mM EDTA, pH 8.0

10% SDS

3.8.2. Genomic DNA extraction

The fungal mycelia was grown in PDB for 6-7 days and then harvested. Total genomic DNA was extracted as described by Kuramae and Izioka (1997). The mycelium was ground into the fine powder under liquid nitrogen and suspended in 700 µL extraction buffer . Upon homogenization, the tubes were incubated for 30 minutes at 65ºC. DNA samples were purified with equal volumes of chloroform: isoamyl alcohol (24:1) mixture (1X), and precipitaded with isopropanol. The tubes were centrifuged at 15400g for 10 minutes and DNA pellets were rinsed with 70% ethanol, air dried, suspended in TE buffer (pH 8.0) and stored at 4° till further use.

3.8.3. Purification of genomic DNA

The extraction of total genomic DNA from the isolated microorganisms as per the above procedure was followed by RNAase treatment. Genomic DNA was

resuspended in 100 µl 1 X TE buffer and incubated at 37$_o$C for 30 min with RNAse (60µg). After incubation the sample was re-extracted with PCI (Phenol: Chloroform: Isoamylalcohol 25:24:1) solution and RNA free DNA was precipitated with chilled ethanol as described earlier. The quality and quantity of DNA was analyzed both spectrophotometrically and in 0.8% agarose gel. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

3.8.4.Agarose gel eletrophoresis to check DNA quality

Gel electrophoresis is an important molecular biology tool. Gel electrophoresis enables us to study DNA. It can be used to determine the sequence of nitrogen bases, the size of an insertion or deletion, or the presence of a point mutation; it can also be used to distinguish between variable sized alleles at a single locus and to assess the quality and quantity of DNA present in a sample.

3.8.4.1. Preparation of DNA samples for electrophoresis

Agarose (0.8%) in 1X TBE buffer was melted, cooled and poured into the gel casting tray with ethidium bromide. Gels solidify in 15-20 min.

3.8.4.2. Run gel electrophoresis for DNA fraction

15µl of sample and 5µl of DNA loading dye mixed properly was loaded in each well of agarose gel (1%).The electrical head of the gel tank was attached firmly and electric supply was applied at constant current 90 mA and voltage 75 volt (BioRAD Power Pac 3000) at least for 90 min. The DNA migrated from cathode to anode. Run was continued until the bromophenol blue had migrated an appropriate distance through the gel. Then electric current was turned off and gel was removed from the tank and examined on UV transilluminator and photographed for analysis.

3.9. ITS PCR analysis

All isolates of *Colletotrichum* and *Pestalotiopsis* were taken up for ITS-PCR amplification. Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. PCR was programmed with an initial denaturing at 94^{o}C for 5 min. followed by 30 cycles of denaturation at 94^{o}C for 30 sec, annealing at 59^{o}C for 30 sec and extension at 70^{o}C for 2 min and the final extension at 72^{o}C for 7 min in a Primus

96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25% bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis.

3.9.1. ITS- PCR primers

The following primers were used to amplify ITS regions

Seq Name	Primer Seq 5'-3'	Mer	TM	%GC
ITS-1	TCTGTAGGTGAACCTGCGG	19	63.9	57
ITS-4	TCCTCCGCTTATTGATATGC	20	61.5	45
CgInt	GGCCTCCCGCCTCCGGGCGG	20	84.4	90

3.9.2. Amplification conditions

Temperature profile, 94^{o}C for 5 min. followed by 30 cycles of denaturation at 94^{o}C for 30 sec, annealing at 59^{o}C for 30 sec and extension at 70^{o}C for 2 min and the final extension at 72^{o}C for 7 min in a Primus 96 advanced gradient Thermocycler.

3.9.3. Sequencing of rDNA gene

The rDNA was used for sequencing purpose. DNA sequencing was done bidirectionally using the ITS primer pairs by SciGenom, Kerala. DNA sequence information was analyzed using bioinformatic algorithms tools e.g. Bioedit, MEGA 4, NTSYSpc as well as the few online softwares. The chromatogram of the DNA sequence was analysed by the software Chromus. All the DNA sequences was edited by using the software BioEdit and aligned with Clustal W algorithms.

3.10. BLAST analysis of the sequences

The DNA sequences were analyzed using the alignment software of BLAST algorithm (http://ingene2.upm.edu.my/Blast, Altschul *et al.*, 1997) for the different characteristic of DNA sequence for the identification of microorganism Identification of microorganism was done on the basis of homology of sequence.

3.11. Submission of rDNA gene to NCBI genbank

The DNA sequences were deposited to NCBI GenBank through BankIt procedure and approved as the ITS sequence after complete annotation and given accession numbers.

3.12. Multiple sequence alignment and Phylogenetic analysis

The sequenced PCR product was aligned with ex-type STRAIN SEQUENCES FROM ncbi Gene Bank and established fungal taxonomy for identification. Sequences were aligned following the Clustal W algorithm (Thompson et al; 1994), included in the Megalign module (DNASTAR Inc.). Multiple alignment parameters used were gap penalty = 10 and gap length penalty = 10. Both of these values are aimed to prevent lengthy or excessive numbers of gaps. The default parameters were used for the pairwise alignment. The use of Clustal W determines that, once a gap is inserted, it can only be removed by editing. Therefore, final alignment adjustments were done manually in order to artificial gaps. Phylogenetic analyses were completed using the MEGA package (version 4.01). Neither gaps (due to indertion-deletion events) nor equivocal sites were considered phylogenetically informative. Hence, complete deletion prevented the use of any of these sites in further analyses. Phylogenetic interference was performed by the UPGMA method (Sneath and Sokal, 1973). Bootstrap tests with 1000 replications were conducted to examine the reliability of the interioir branches and the validity of these trees obtained. Phylogenetic analyses were conducted in MEGA 4 as described by Tamura et al., 2007.

3.13. RAPD PCR analysis

For RAPD, random primers were selected (Table-1). PCR was programmed with an initial denaturing at 94oC for 4 min. followed by 35cycles of denaturation at 94oC for 1 min, annealing at 36oC for 1 min and extension at 70oC for 90 s and the final extension at 72oC for 7 min. in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25 % bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination by horizontal electrophoresis.

3.13.1. RAPD primers

The following primers were used for RAPD analysis in the study

Seq Name	Primer Seq 5'-3'	Mer	TM	%GC
OPA-1	CAGGCCCTTC	10	38.2	70
OPD-6	GGGGTCTTGA	10	32.8	83
OPD-12	TGAGGGGAGA	10	36.9	60

3.13.2. Amplification conditions

Temperature profile, 94oC for 4 min followed by 35cycles of denaturation at 94oC for 1 min, annealing at 36oC for 1 min and extension at 70^{o}C for 90 s and the final extension at 72oC for 7 min in a Primus 96 advanced gradient Thermocycler.

3.13.3. Analysis of RAPD band patterns

RAPD band patterns were initially assessed by eye and isolates were grouped according to their shared band patterns.

3.13.4. Scoring of individual bands

Two methods of scoring bands were assessed. The first method involved scoring bands using the computer programme NTSYSPc and the second method was to score the number of shared bands (i.e. bands of equal size) on a gel by eye. For both methods, photographs of the gels were scanned into a computer and saved as graphics files.

3.13.5. Reconstruction of the phylogenetic tree

As with sequence data, RAPD data can be analysed in a number of different ways. The simplest form of analysis is to group isolates with identical band patterns for a given primer. More complex analyses involve cladistic analysis of data and reconstruction of the phylogenetic tree.

3.13.5.1. UPGMA method

The image of the gel electrophoresis was documented through Bio-Profil Bio-1D gel documentation system and analysis software. All reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed *InSilico* into similarity matrix using NTSYSpc (Numerical Taxonomy System Biostatistics, version 2.11W). The SIMQUAL program was used to calculate the Jaccard‘s coefficients. The RAPD patterns of each isolate was evaluated, assigning character state “1” to indicate the presence of band in the gel and “0” for its absence in the gel. Thus a data matrix was created which was used to calculate the Jaccard similarity coefficient for each pair wise comparison. Jaccard coefficients were clustered to generate dendograms using the SHAN clustering programme, selecting the unweighted pair-group methods with arithmetic average (UPGMA) algorithm in NTSYSpc.

3.14. Denaturing Gradient Gel Electrophoresis (DGGE)

3.14.1 PCR amplification of genomic DNA of the isolates for DGGE analysis

Denaturing Gradient Gel Electrophoresis was performed according to the method of Zhao et al; 2006. 18S DNA (200bp with GC clamp) was amplified with the forward primer containing GC clamp at 5' end) F352T : 5'- CGC_CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG TGG C-3' and 519r: 5'- ACC GCG GCT GCT GGC AC-3') in 25µl of reaction mixture containing 1X PCR buffer, 2.5mM MgCl2 (Bangalore Genei, India), 100 ng of the template DNA,25.0pmol each of the forward and reverse primers, 250µM each of the dNTPS, and 1U of Taq DNA polymerase (Bangalore Genei, India). The touchdown PCR program was performed which consisted of an initial denaturation at 95°C for 5min, followed by 6 cycles of 95°C for 1min,65°C for 1min, and 72°C for 1min, in which the annealing temperature was reduced by 0.5°C per cycle from the preceding cycle, and then 24 cycles of 95°C. Perpendicular DGGE was performed with "the decode universal mutation detection system" (Bio Rad laboratories, USA).

A uniform gradient gel of 0% to 100% denaturant was prepared which was changed several times so as to optimise suitable concentration and finally 20% to 60% denaturant was found optimal for the best result.

3.14.2. Denature Gradient Gel Electrophoresis of the PCR products

3.14.2.1. Reagents and solutions required for DGGE analysis

40% Acrylamide:bisacrylamide (37.5:1)

50X DGGE/TAE buffer solution

Trizma-Base:	484.4g
Sodium-Acetate:	272.0g
Trisodium EDTA	37.2g
H_2O	2 litres

pH 7.40 adjusted with about 230ml of glacial acetic acid

Preparation of Denaturants

100% Denaturant:

Urea	42.0g
38.5% Acrylamide (makes a 6.5% gel)	16.9ml
50X DGGE/TAE	2.0 ml
Formamide	40.0ml

Filled up to 100ml with distilled water.

0% Denaturant:

38.5% Acrylamide (makes a 6.5% gel)	16.9ml

50X DGGE/TAE	2.0 ml
Ammonium persulphate	10% (w/v)
TEMED	20µl

3.14.2.2. Creating the gel sandwich (DCode System BioRad)

Large glass plates were cleaned with soap and a soft sponge and rinsed with tap water. After drying, they were cleaned with 96% ethanol. Both 1mm spacers were also cleaned with 96% ethanol and placed on the large glass plates. The clamps were scrwed to the sides of the sandwich, in order to be sure that the spacers, 2 glass plates and especially the glass plates were aligned at the bottom side of the sandwich and placed in the holder. The clamps were unscrewed and the alignment card slid between the glass plates to align the spacers. The clamps were screwed and the alignment of the glass plates were checked. Then the sandwich was placed on top of the rubber gasket and the handles pressed down.

3.14.2.3. Preparation of the gel

One tube of APS 10% per gel was prepared. The tubing needle was replaced with a new one, the screw between the compartments was opened and the compartments rinsed with water using pump at a uniform speed. The system was completely drained and flushed with compressed air. The gel solutions were prepared as required. Stacking gel was also prepared according to the following:

UF solution	UF (%)	Acrylamide/ bis (%)	Volume UF solution (ml)	Volume APS 10% (µl)	Volume TEMED (µl)
Low	30-45	6	13	78	6
High	60	6	13	78	6

APS and TEMED was added to the high and low solutions according to the table, stirred gently by hand and proceeded immediately for pouring the high concentration solution in the compartment closest to the outlet of the gradient mixer and the low concentration solution in the other compartment by the delivery system. The whole system was kept for polymerization.

3.14.2.4. Running a gel

Fresh 0.5X TAE buffer was added to the buffer tank to the mark "Fill". The DCode^{TM} Universal Mutation Detection System (Bio-Rad) was switched on at least 60 minutes before electrophoresis, so that the buffer can heat up to 60°C. After 2-3 hours of

polymerization, the comb was removed carefully and the bottom of the sandwich was rinsed with tap water to remove non-polymerized gel. The sandwich was set in the sandwich-holder. A dummy sandwich was also set at the other side to get a closed upper buffer compartment. (A dummy consists of a large and small glass plate stuck together with no spacers in between). The DCode^{TM} was then switched off and the lid taken off after 1 min. The sandwich holder was then slided into the buffer tank, with the red dot of the cathode at the right side. The DCode^{TM} pump and the stirrer underneath the tank were switched on (300 rpm) until the samples were loaded.

3.14.2.5. Staining of gels and photography

Before taking DGGE units out from the tank, the run-evaporated H2O was replaced up to the marked level. Carefully the DGGE unit was dismantled. The ethidium bromide stain was added into a tray with 50X TAE buffer and the gel was stained for 5 minutes and destained with running buffer. The gel was photographed under UV-transilluminator.

3.14.2.6. Data Analysis

3.14.2.6.1. Scoring of individual band

Two methods of scoring bands were assessed. The first method involved scoring bands using the computer programme BioProfil 1D and the second method was to score the number of shared bands (i.e. bands of equal size) on a gel by eye. For both methods protograph the gels were scanned into a computer and saved as graphic files.

3.14.2.6.2. UPGMA analysis of the DGGE bands.

Variability among the different groups of isolates were detected on the basis of the banding pattern obtained on denature gradient gel. All reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed *in silico* into similarity matrix using NTYSYpc (Version 2.11W) as in case of RAPD analysis. However, a more complex analysis involved cladistic analysis of data and reconstruction of phylogenetic tree. A two (2-D) and three dimension (3-D) principal component analysis was constructed to provide another means and test the relationship among different tested groups using EIGEN programme (NTSYS-PC).

3.15. Assessment of disease caused by fungal pathogens on som plants

3.15.1. Detached leaf inoculation

A detached leaf inoculation technique as described by Dickens and Cook (1989) was followed for artificial inoculation of leaves. Fresh, young, fully expanded som leaves detached from plants were placed in trays lined with moist blotting paper. Wounds were made on adaxial surface of each leaf with 26 G½ needle and inoculated with 20µl droplets of spore suspension (1.2 X 10^6 conidia ml^{-1}) of the fungus (prepared from 14 days old culture in PDA). Spore suspension was placed (2-4 drops $leaf^{-1}$) on the adaxial surface of each leaf with a hypodermis syringe on the wounds. In control sets drops of sterile distilled water were placed on the wounded leaves. Each tray was covered with a glass lid and sealed with petroleum jelly in order to minimize drying of the drops during incubation.

Percent drops that resulted in lesion production were calculated after 48, 72 and 96 hours of incubation as described by Chakraborty and Saha (1994). Observations were made based on 50 inoculated leaves for each treatment in average of three separate trials.

3.15.2. Whole Plant inoculation

Whole plant inoculation was carried out essentially as described by Mathur *et al* (2000) with minor modifications. The fungus was grown in PDA for 14 days at 30°±2°C and spore suspension was prepared (1.2 X 10^6 conidia ml^{-1}). Tween-20 was added @ 2ml l^{-1} to facilitate adhering of the spores to leaf surface. 2 year old plants were spray-inoculated with an atomizer @ 100ml per plant so as to wet both ventral and dorsal surfaces. The plants were immediately covered with polythene bags so as to maintain high relative humidity and kept overnight. Next day, the polythene bags were removed and transferred to glass house benches and maintained at 30°±2°C.

The disease severity on plant leaves was recorded using a 0-5 rating scale (Lakshmi et al 2011), where 0 = no infection; 1 = up to 5%; 2 = 6-10%; 3 = 11 20%; 4 = 21-50% and 5 = > 50% leaf area affected by the disease. Based on this numerical ratinga Percent Disease Index (PDI) was calculated using the formula:

PDI = [total numerical ratings/(number of leaves examined × max rating scale)]× 100.

Results were always computed as the mean of observations of 25 well-established and branched 7 month old som plants in average of three separate experiments.

3.16. In vitro testing for antagonism to fungal pathogens

3.16.1. Antifungal test of Plant growth promoting rhizobacteria (PGPR)

The obtained PGPR isolates were evaluated against leaf pathogen *C. gloeosporioides* and *P. disseminata* in dual culture using NA medium. The bacteria were streaked on one side of the Petri plate and 4mm fungal pathogen block was placed at the other side of theplate, incubation was undertaken for 5-7 days at 28º±2ºC and inhibition zone towards the fungal colony in individual plate was quantified. Results were expressed as mean of percentage of inhibition of the growth of the pathogen in presence of the bacterial isolates. For each test three replicate plates were used. Those bacteria, which were antagonistic to *C. gloeosporioides* and *P. disseminata*, were selected for further evaluation and identification.

3.16.2. Antifungal test of Plant growth promoting fungus (PGPF)

The efficacy of PGPF (*Trichoderma* sp.) isolates was tested *in vitro* for inhibiting growth of the pathogen (*C. gloeosporioides* and *P. disseminata*) in dual culture using PDA. Each fungal isolate was placed at one side of the agar plate about 1cm away from the edge and a 4mm diameter block of the pathogen, taken from growing edge replicate plates were used. The plates were incubated for 7 days (depending upon the growth of the pathogen) at 28ºC and inhibition zone towards the fungus colony in individual plate was quantified. Results were expressed as mean % of inhibition in presence of the fungal isolate.

3.17. Mass multiplication and application of bioinoculants and pathogen

3.17.1 Arbuscular Mycorrhizal Fungi (AMF)

3.17.1.1. Isolation of AMF spores

Spores of arbuscular mycorrhizal fungi were isolated from rhizosphere soil of som by wet sieving and decanting method (Gerdemann and Nicolson, 1963). Approximately 250 g of soil was suspended in 1 L water. Heavier particles were allowed to settle for a few seconds and the liquid was decanted through sieves of decreasing size (BS 60, BS 80, BS 100, BS 150 and BS 200). Pores are fine enough to remove the larger particles of organic matter, but coarse enough to allow the desired spores to pass through. The suspension that passed through these sieves was saved and stirred to resuspend all particles. The heavier particles were allowed to settle for a few seconds and the liquid decanted again through the sieve and spores collected by fine brushes and were kept in different Petri plates according to their size and colours. Moreover for further observations or purification of AMF spores sucrose gradient centrifugation method was used. In sucrose gradient centrifugation (Daniels and Skipper, 1982),

spores and minimal amount of organic particles were further purified by suspending sieving in 40% sucrose solution and centrifuging at 2000 rpm (approximate 370 x g) for 1 minute. The supernatant (with spores) was passed through a sieve of 400 mesh and rinsed with distilled water to remove sucrose residue.

With the help of a simple microscope (20X) parasitized spores, plant debris etc were separated. Spores were sonicated at 30 Hz for two minutes to remove the debris adhered to the spores then clean spores were stained with Melzar's reagent (50% aqueous solution of chloral hydrate with 2.5-3.75% potassium iodide and 0.75-1.25% iodine) and studied microscopically. For further use, the AMF spores were stored in Ringer's Solution (8.6g NaCl, 0.3g KCl, 0.33g $CaCl_2$ in 1 L of boiled distilled water) at -15ºC to - 20 ºC or in sterile distilled water. Identification of genera and species was done microscopically using the specific spore characters such as size, colour, shape, wall structure, surface ornamentation and bulbous suspensor by using identification manuals (Trappe, 1982; Schenck and Perez, 1990).

3.17.1.2 Histopathology of som roots

Fungal association of AM fungi within the root tissues was observed according to Philips & Hayman (1970). Young roots from mandarin plants were dug out manually. Roots were cut into 1cm or smaller pieces and washed in tap water gently to free them from soil particles. It was boiled in 2% KOH in hot water bath for 1 hour. The KOH was decanted and the roots washed with water for 2-3 times. 1% HCL was added and kept for 30 minutes. After decanting the HCL the sample was washed thrice in tap water and cotton blue, lactic acid and glycerol was added in the ratio 1:1:1 to stain the internal structures of AMF inside the root segments i.e. arbuscules, vesicles, auxiliary cells, and boiled in water bath for 1 hour. The excess stain was decanted and sample placed in 50% glycerol for destaining. The roots were then crushed under pressure in slide and covered with cover slip for microscopic observation. Percent root colonization was determined following the method of Giovanetti and Mosse (1980).

3.17.1.3 Mass multiplication of AMF

AMF spores were multiplied in roots of maize as host plant. AMF spores were isolated from rhizosphere of all eight morphotypes of som plant using decanting and sieving method as described earlier. The mass of spores were washed with distilled water several times to remove the adhered debris. Filter paper was cut into small bits about the size of 1 cm. With the help of fine tweezers, 45-50 AMF spores were placed in the filter paper bits. They were then carefully placed onto the roots of the 7-10 days

old host seedling in plastic pots (12inch) having autoclaved soil to discard the presence of other fungal propagules. Maize plants were grown both in the field and pots. After 45 days, the presence of spores was verified and inocula were prepared by mixing the chopped roots of maize plants with the potted soil where extra radical spores of AMF were present. Approximately > 175 spores / 100gms could be considered as potent inocula for application.

3.17.2 Plant Growth promoting Fungi (PGPF)

3.17.2.1. Selection of PGPF

One isolate of *Trichoderma harzianum* (BRHS/480) and another isolate of *Trichoderma asperellum* (RHS/S569) were selected as Plant growth promoting fungi based on their performance as potential growth promoters as well as their biocontrol efficiency in field grown crops (Sunar *et al.*, 2014)

3.17.2.2 Mass multiplication

3.17.2.2.1 Wheat bran media

Inoculum of *Trichoderma asperellum* and *Trichoderma harzianum* were prepared by inoculating wheat bran (sterilized) with 5 mm disc of the fungus and incubating at 28ºC for 10 days. To each pot of soil (2000 g), 10 g of the wheat bran colonized by *T.asperellum* and *T. harzianum* was mixed to give a concentration of 105 cfu / g of soil as described by Chakraborty *et al.*, (2003).

3.17.2.2.2 Tricho-compost

Six layers of compost materials (each layer about 25 cm thick) was made. 3 parts cellulosic waste (rice straw, grass, corn stalk, spent mushroom substrate) and 1 part mixture of leguminous plant materials (Mungbean, Peanut, Soybean) and animal manure was mixed. Each layer of piled compost materials was sprinkled with 30 litres of Tricho inoculants solution. Additional water is sprinkled to keep the compost heap moist. It was covered with plastic sheet or sack to increase temperature and prevent too much water in case of rainfall. Compost heap was turned from top to bottom after two weeks. The Tricho compost was ready for harvest four weeks after preparation. The compost was stored in sacks or applied directly into the soil.

3.17.3. Plant Growth promoting Rhizobacteria (PGPR)

One isolate of *Bacillus pumilus* (BRHS/C1) and one isolate of *Bacillus altitudinus* (BRHS/S73) were selected as Plant growth promoting rhizobacteria based on their

performance as potential growth promoters as well as their biocontrol efficiency in field grown crops (Sunar *et al.*, 2013)

3.17.3.1. Selection of PGPR

3.17.3.2 Mass multiplication

3.17.3.2.1 Soil drench

The bacteria were grown in NB for 48 h at 28ºC and centrifuged at 12,000rpm for 15 minute. The pellet obtained was suspended in sterile distilled water. The optical density of the suspension was adjusted using UV-VIS spectrophotometer following method to obtain a final density of 3 $X10^6$ cfu ml^{-1}.The bacterial suspension was applied to the pots during transplantation of seedlings. Applications were done @ 100 ml per pot at regular interval of one month for three months subsequently. The rhizosphere of two year‘s old potted plant was inoculated twice at an interval of 20-25 days.

3.17.3.2.2 Foliar spray

The bacterial pellet suspended in sterile distilled water at a concentration of 3x106 cfu ml-1 after the addition of a few drops of Tween -20 was sprayed until run off on the foliar part of the ten year old bushes after pruning. The spraying was done forth nightly till the new shoots started appearing.

3.17.3.2.3 Talc based formulation

Ten g of carboxy methyl cellulose sodium salt (Himedia) was mixed with one kg of talcum powder and pH was adjusted to 7.0 by adding calcium carbonate. It was then sterilized twice for 30 min each. The bacterium was first grown in nutrient broth and after 48 h the actively growing cells in log phase were harvested by centrifugation at 21 000 g, and aqueous suspension was made to achieve a concentration of $3x10^9$ CFU ml^{-1} which was determined spectrophotometrically. To 1 kg of sterilized talcum powder 400 ml of bacterial inoculum was added and mixed well under sterile condition. The talc mix was dried under shade to bring moisture to less than 20%. The formulation was packed in milky white colour polythene bags to eliminate UV exposure, sealed and stored at room temperature for future use. The talcum based formulation was applied in the field at the rate of 100 g per pot (12 $x10^{10}$ bacterial cells).

3.17.4 Vermicompost preparation

Vermicompost was prepared in plastic beds using organic waste materials collected from the local area. 15-20 cm layer of this waste was covered with another 2-3 cm of

dried aquatic plants. *Eisenia foetida*, the earthworm used for vermicomposting was added on the top. The final top layer was made of dried cow dung and the vermin bed was sealed with plastic cover. This set was kept undisturbed for 15-20 days, after which the bed was stirred and shaken to release the organic gas produced during vermicomposting process and for proper mixing of the materials. The compost was ready after 40-45 days when it turned into black light weight powder with no odour. After its completion the earthworms are separated from the final product and the manure was dried and sieved for further use. This vermicompost was applied to the field at the rate 200g per pot.

3.17.5 Application of different bioinoculants under pot and field condition

In case of pot treatment, initially Vermicompost is added to the soil @ 200g/pot. Then AMF spores were added to the roots of som sapling using filter paper disc. After 15 days of treatment, *T. asperellum* in wheat bran medium was added to the pots. Two weeks after application of PGPF, foliar spray as well as soil drench application of PGPR was done for 15 days at 5 days interval. In case of joint treatment with all three bioinoculants, they were added to the pots sequentially but in case of dual treatments (AMF+PGPF, AMF+PGPR, PGPF+PGPR) application was done accordingly.

For field inoculation, chopped maize roots colonized with dominant spores of *G. constrictum* (AMF) were applied in the root rhizosphere following transplantation in the field from nursery- grown 7 month old plants. One month following application of AMF, root colonization status was examined. Then mass multiplied *T. asperellum* made with wheat bran was applied in soil. Two weeks after application of *T. asperellum* (PGPF), further soil application of talc based formulation as well as foliar spray of *B. altitudinus* (PGPR) was done. Growth parameters were finally recorded after 6 months of application of last treatment.

3.17.6. Inoculum preparation of pathogen

The leaf blight pathogen, *Colletotrichum gloeosporioides* and the grey blight pathogen *Pestalotiopsis disseminata* were grown in 100ml PDA medium for 7-10 d till sporulation occurred. The spores were then scraped off from the surface of the media with the help of inoculating needle and the spores were collected insterile distilled water. The spore suspension containing3×10^4 spores/ml with 0.01% Tween 20 was sprayed on to the treated as well untreated potted plants and covered with plastic bags for 48 h.

3.18. *In vivo* assessment of plant growth promotion

3.18.1. Assessment of plant growth following application of bioinoculants

Plant growth promotion was recorded after 30 and 60 d of application of bioinoculants in potted plants and after four months in the field grown plants. The growth parameters such as number of leaves, branches and height were observed.

3.18.2. Assessment of disease severity.

The disease severity on plant leaves was recorded using a 0-5 rating scale (*Lakshmi et al 2011*), where 0 = no infection; 1 = up to 5%; 2 = 6-10%; 3 = 11 20% 4 = 21-50% and 5 = > 50% leaf area affected by the disease. Based on this numerical rating a Percent Disease Index (PDI) was calculated using the formula:

PDI = [total numerical ratings/(number of leaves examined × max rating scale)]× 100.

3.18.3. Assessment of soil phosphate mobilization

3.18.3.1. Extraction of Soil phosphate

Soil sample (1g) was air dried and suspended in 25 ml of the extracting solution (0.025N H_2SO_4, 0.05N HCl) to which activated charcoal (0.01g) was also added, shaken well for 30 min on a rotary shaker and filtered through Whatman No. 2 filter paper (Mehlich, 1984). Quantitative estimation of phosphate was done following ammonium molybdate-ascorbic acid method as described by Knudsen and Beegle (1988).

3.18.3.2. Estimation of soil phosphate

For estimation, 2 ml aliquot of the soil extract was transferred to test tube along with the transfer of 2 ml aliquots of each of the working standards. Then 8 ml of the colorimetric working solution (25 ml conc. ammonium paramolybdate solution, 10 ml ascorbic acid soln., final volume- 1000ml) was added to each test tube and mixed thoroughly. They were allowed to wait for 20 mins for colour development. Finally, % transmittance of all standards and samples on a colorimeter with wavelength set at 882 nm was recorded.

3.19. Extraction and Assay of defense enzyme activities after application of bioinoculants

3.19.1. ß-1, 3- glucanase (E.C. 3.2.3.39)

Extraction of ß-1,3- glucanase (E.C. 3.2.3.39) was done following the method described by Pan et al.. (1991). Mandarin root and leaf samples (1g) were crushed in

liquid nitrogen and extracted using 5ml of chilled 0.05 M sodium acetate buffer (pH 5,0) by grinding at 4 ºC using mortar and pestle. The extract was then centrifuged at 10000 rpm for 15 min at 4 ºC and the supernatant was used as crude enzyme extract.

Estimation of the ß-1,3-glucanase was done by following the Laminarin dinitrosalicylate method (Pan et al., 1991): The crude enzyme extract of 62.5µl was added to 62.5 µl of laminarin (4%) and then incubated at 40ºC for 10 minutes. The reaction was stopped by adding 375µl dinitrosalicylic reagent and heating for 5 min in boiling water bath. The resulting colored solution was diluted with 4.5 ml of water, vortexed and absorbance was recorded at 500nm. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. The enzyme activity was expressed as µg glucose released min^{-1} g^{-1} fresh tissues

3.19.2. Chitinase (E.C. 3.2.1.14)

Extraction of chitinase (E.C. 3.2.1.14) was done by following the method described by Boller and Mauch (1988) with modifications. 1g root and leaf sample from mandarin plants were crushed in liquid nitrogen and extracted using 5ml of chilled 0.1M Sodium Citrate buffer (pH 5). The homogenate was centrifuged for 10minutes at 12,000rpm and the supernatant was used as enzyme source.

Chitinase activity was measured according to the method described by (Boller and Mauch, 1988). The assay mixture consisted of 10µl Na-acetate buffer (1M) pH 4, 0.4ml of enzyme solution, 0.1ml of colloidal chitin (1mg). Colloidal chitin was prepared as per the method of (Roberts and Selitrennikoff, 1988). After 2h of incubation at 37 ºC the reaction was stopped by centrifugation at 10,000g for 3minutes. An aliquot of supernatant(0.3ml) was pipetted into a glass reagent tube containing 30µl of potassium phosphatebuffer (1M) pH7.1 and incubated with 20µl of (3%w/v) desalted snail gut enzymeHelicase (Sigma) for 1hour. After 1h, the pH of the reaction mixture was brought to 8.9by addition of 70µl of sodium borate buffer (1M) pH9.8. The mixture was incubated in a boiling water bath for 3minutes and then rapidly cooled in an ice water bath. After addition of 2ml of DMAB (ρ-dimethyl aminobenzaldehyde) reagent, the mixture was incubated for 20 min at 37 ºC. There of absorbance value at 585nm was measured using a UV-VISspectrophotometer. N-acetyl glucosamine (GlcNAc) was used as standard. The enzyme activity was expressed as µg GLcNAc min^{-1} mg^{-1} fresh tissues.

3.19.3. Phenylalanine ammonia lyase (PAL) (E.C. 4.3.1.5)

Extraction of PAL (E.C. 4.3.1.5) was done by following the method described by Chakraborty *et al.* (1993) with modifications. 1gm root and leaf sample was crushed in 0.1M sodium borate buffer pH 8.8 (5ml/gm) with 2mM of ß mercaptoethanol in ice cold temperature. The slurry was centrifuged in 15000 rpm for 20 minutes at 4ºC. Supernatant was collected and after recording its volume, was immediately used for assay or stored at -20ºC.

Phenylalanine ammonia lyase activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The reaction mixture contained 0.3 ml of 300µM sodium borate (pH 8.8), 0.3 ml of 3 µM L- phenylalanine and 0.5ml of supernatant in a total volume of 3ml. Following incubation for 1 h at 40 ºC the absorbance at 290nm was read against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as µg cinnamic acid produced in 1 min g -1 fresh weight of tissues.

3.19.4.Peroxidase (E.C. 1.11.1.7)

For the extraction of peroxidase (E.C.1.11.1.7) the plant tissues were macerated to powder in liquid nitrogen and extracted in 0.1 M Sodium borate buffer (pH 8.8) containing 2 mM ß mercaptoethanol under ice cold conditions, the homogenate was centrifuged immediately at 15000 rpm for 20 minutes at 4 ºC. After centrifugation the supernatant was collected and after recording its volume was immediately used for assay or stored at -20 ºC (Chakraborty *et al..*, 1993).

For determination of peroxidase activity, 100µl of freshly prepared crude enzyme extract was added to the reaction mixture containing 1 ml of 0.2 M sodium phosphate buffer (pH 5.4), 100µl of 4mM H2O2, 100 µl O-dianisidine (5mg ml-1 methanol) and 1.7ml of distilled water. Peroxidase activity was assayed spectrophotometrically at 460 nm by monitoring the oxidation of O- dianisidine in presence of H_2O_2 (Chakraborty *etal..*, 1993). Specific activity was expressed as the increase in absorbance at 460 nm g^{-1} tissue/ min^{-1}.

3.19.5. Isozyme analysis of peroxidase

Polyacrylamide gel electrophoresis (PAGE) was performed for isozyme analysis of peroxidase. Extract for isozyme analysis was prepared by crushing 1 g of leaf tissue in a mortar and pestle in 2 M sodium phosphate buffer (pH 7.0) in ice cold condition as described by Davis (1964) and used immediately for the isozyme analysis.

3.19.5.1. Preparation of the stock solution

Solution A: Acrylamide stock solution (Resolving gel)

For the preparation of acrylamide stock solution for resolving gel 28 g of acrylamide and 0.74 g of N‘ N‘ methylene bisacrylamide was dissolved in 100 ml of distilled water. The stock solution was filtered with Whatman No. 1 filter paper and stored at 4°C in dark bottle.

Solution B: Acrylamide stock solution (stacking gel)

For the preparation of acrylamide stock solution for stacking gel 10 g of acrylamide and 2.5 g of N‘ N‘ bisacrylamide was dissolved in 100 ml of distilled water. The stock solution was filtered and stored at 4°C in dark bottle.

Solution C: Tris- HCl (Resolving gel)

36.6 g of Tris base was mixed with distilled water and 0.25 ml of N, N, N‘, N‘-tetramethyl ethylene diamine (TEMED) was added. The pH was adjusted to 8.9 with concentrated HCL. The volume of the solution was made up to 100 ml with distilled water. The solution was then stored at 4°C for further use.

Solution D: Tris- HCl (Stacking gel)

5.98 g of Tris base was mixed with distilled water and 0.46 ml of TEMED and the pH was adjusted to 6.7 with concentrated HCl. The volume of the solution was made up to 100 ml with distilled water. The solution was stored at 4°C for further use.

Solution E: Ammonium persulphate solution (APS)

Fresh solution of APS was prepared by dissolving 0.15 g of APS in 10 ml of distilled water

Solution F: Riboflavin solution

Fresh solution of Riboflavin was prepared by dissolving 0.4 mg of riboflavin in 10 ml distilled water. The solution was kept in dark bottle to protect from light.

Solution G: Electrode buffer

Electrode buffer was prepared freshly by dissolving 0.6 g of Tris base and 2.9 g glycine in 1 L of distilled water.

3.19.5.2. Preparation of gel

For the polyacrylamide gel electrophoresis of peroxidase isozymes mini slab gel was prepared. For slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any trace of grease and then dried. 1.5 mm thick spacers were placed between the glass plates on three sides and these were sealed with high vacuum grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. 7.5 % resolving gel was prepared by mixing solution A: C: E:

distilled water in the ratio of 1: 1: 4: 1 by pipette leaving sufficient space for (comb + 1 cm) the stacking gel. This resolving gel was immediately over layered with water and kept for polymerization for 2 hours. After polymerization of the resolving gel was complete, over layer was poured off and washed with water to remove any unpolymerized acrylamide. The stacking gel solution was prepared by mixing solutions B: D: F: distilled water in the ratio of 2: 1: 1: 4.

Stacking gel solution was poured over the resolving gel and comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30- 45 minutes in strong sunlight. After polymerization of the stacking gel the comb was removed and washed thoroughly. The gel was now finally mounted in the electrophoretic apparatus. Tris- Glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble, trapped at the bottom of the gel, was removed very carefully with a bent syringe.

3.19.5.3. Sample Preparation

Sample (32 µl) was prepared by mixing the sample enzyme (20 µl) with gel loading dye (40 % sucrose and 1 % bromophenol blue in distilled water) in cyclomixture in ice cold condition. All the solutions for electrophoresis were cooled. The samples were immediately loaded in a predetermined order into the bottom of the wells with a microlitre syringe.

3.19.5.4. Electrophoresis

Electrophoresis was performed at constant 15 mA current for a period of 3 - 4 h at 4°C until the dye front reached the bottom of the gel.

3.1.95.5. Fixing and Staining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally stained. Staining of the gel was performed following the method of Reddy and Gasber (1973). The gel was incubated in the aqueous (80 ml) solution of Benzidine (2.08 g), Acetic acid (18 ml), 3 % H_2O_2 (100 ml) for 5 minutes. The reaction was stopped with 7 % Acetic acid. After the appearance of clear blue colored bands, analysis of isozyme was done immediately.

3.19.6. Extraction and estimation of phenol content

3.19.6.1. Extraction of phenol

Phenol was extracted from the fresh young leaves and roots following the method of Mahadevan and Sridhar (1982). One g of sample were cut into pieces and

immediately immersed in 10 ml of boiling alcohol. After 15 minutes of boiling it was cooled and crushed in mortar using pestle thoroughly at room temperature. The extract was filtered through Whatmann No. 1 filter paper. Final volume was adjusted with 80 % ethanol. The whole extraction of phenol was done in dark to prevent light induced degradation of phenol.

3.19.6.2. Estimation of Total phenol

Total phenol content was estimated by Folin Ciocalteau‘s reagent, following the method of Mahadevan and Sridhar (1982). To 1 ml of the alcoholic extract, 1 ml of 1 N Folin Ciocalteau‘s reagent followed by 2 ml of 20 % sodium carbonate solution was added in a test tube. The test tube was shaken and heated on a boiling water bath for 1 minute. After cooling, the volume of the reaction mixture was raised to 25 ml. Absorbance of the blue colored solution was measured at 650 nm. Quantity of total phenol was estimated using caffeic acid as standard.

3.19.6.3. Estimation of Ortho-phenol

O-dihydroxy phenol was also estimated following the method of Mahadevan and Sridhar (1982). 1 ml of alcoholic extract was mixed with 2 ml of 0.05 N HCl, 1 ml of Arnow‘s reagent ($NaNO_2$ - 10 g, Na_2MoO_4 - 10 g, distilled water - 100 ml) and 2 ml of 1 N NaOH and mixed thoroughly at room temperature following which the volume of the reaction mixture was raised to 10 ml. Absorbance of the colored solution was recorded at 515 nm. Quantity of the O- dihydroxy phenol was estimated using caffeic acid as standard.

3.20. Analysis of antifungal compounds

3.20.1. Collection of leaf diffusates and their bioassay

Leaf diffusates were obtained by a modified drop diffusate technique of Muller (1958). Forty young leaves, each of 8 different morphotypes, were collected from green house, washed and placed on moist filter paper in separate trays. Wound was made in the leaves using needle and spore suspension of *C. gloeosporioides* was placed on the wounds using Pasteur pipette and incubated for 2 days. In case of control, distilled water was placed on the wounds in place of spore suspension. Drops of spore suspension were collected from each leaf of the eight morphotypes separately, centrifuged and supernatants collected. These were passed through

sintered glass filter and their biological activities were assayed on spore germination and appressoria formation (Chakraborty *et al*., 1995).

3.20.2. HPLC analysis of phenolic compounds

Fresh leaves of som plant were chopped into pieces and soaked overnight in methanol in the ratio 1:3 (w/v), filtered through Buckner's funnel and the solvent was evaporated using lyophilizer as described by Pari and Latha (2004). The dried powder was finally mixed in HPLC graded methanol and stored at 4°C for further analysis. HPLC analysis of phenolic compounds present in the extracts was done using SPD-10A VP Shimadzu UV-VIS Detector. A flow rate of 1 mL/min, and gradient elution of acetonitrile-water-acetic acid (5:93:2, v/v/v) [solvent A] and of acetonitrile-water-acetic acid (40:58:2, v/v/v) [solvent B], 0– 50 min solvent B from 0 to 100%; and injection volume of 20 µl were applied; whereas the separation of compounds was monitored at 280 nm (Pari *et al.*, 2007).

3.21. Scanning Electron Microscopy

Spores of fungal pathogens were examined under scanning electron microscopy (SEM). Selected fungal spores were sonicated under 35 MHz to followed by washing five times in sterile distilled water, surface disinfected with 4% (wt/vol) chloramine-T and 300 ppm of streptomycin for 1 h, and then rinsed a further five times in sterile distilled water and were stored in eppendorf‟s tube in room temperature. Each sample was placed within separate aluminium "disc cup" (20 mm diam x 5 mm deep). Each sample was lifted from the bottom of the specimen dish with fine forceps and was positioned upright in a disc cup. The samples were then dried. All dried samples were mounted on double-sided tape affixed to SEM specimen mounts and were subsequently sputter-coated with gold. Gold coated samples were examined with a Philips 505 scanning electron microscope operating at 9.5-r5 Kev.

3.22. Transmission Electron Microscopy

3.22.1. Specimen preparation

3.22.1.1. Fixation

Control and inoculated root samples (1-2 mm) were excised in 0.1M sodium phosphate buffer pH 7.4. They were immediately transferred to 2.5% Glutaraldehyde in eppendorf tubes for 2-12 hours at room temperature.

3.22.1.2. Dehydration

Dehydration was done in ascending grades of alcohol at intervals of 30 mins in 4° C (30%, 50%, 70%, 80%, 90%) and two changes in absolute alcohol at 1 hr interval each at 4° C in PLT-272(M) Fume Hood (Tanco).

3.22.1.3. Infiltration

Infiltration was done twice in LR White resin (London Redin Co. Ltd) in absolute alcohol (1:1) for 1 hr each at 4° C.

3.22.1.4. Embedding

The samples were dipped in LR White and kept overnight at 4° C. They were keptat room temperature for 3 hrs. A fresh change of LR white was done and kept at 56° C for36 hrs.

3.22.2. Viewing preparation

3.22.2.1. Trimming

Moulds containing the samples were roughly trimmed with a block trimmer (Reichert TM60) fitted with a rotating milling cutter

3.22.2.2. Sectioning

A series of thick sections of the selected blocks were cut with Belgium glass strips in microtome (Leica EM UC7) to observe under an optical microscope. These semithin sections are stained with 1% aqueous toluidine blue solution. These sections can be viewed in light microscope.

3.22.3. Immunogold labeling

Ultrathin sections (60nm) were cut with fresh Belgium glass strips and picked up in nickel grids (100 mesh) for immunogold labeling.

3.22.3.1. Primary antibody

The grids containing ultrathin sections were floated in blocking solution containing 2% skimmed milk agar for 30 min. Primary antibody was diluted in 1% fish gelatin in the ratio 1:20. Grids were incubated the PAbs for 24 hrs at 4° C. Grids were washed on drops (100 µl) of fish gelatin pipetted on to parafilm 10X2 min.

3.22.3.2. Secondary antibody

Grids were incubated with anti-rabbit IgG (Whole Molecule) gold antibody produced in goat affinity isolated antibody (Sigma-G7402) diluted in 1:5 in fish gelatin at room temp for 3 hrs.

3.22.4. Staining

Sections were stained with 2% uranyl acetate for 15 min. The sections were washed in double distilled water. Post stain was done in 0.2% lead acetate for 5 min. Washed again in double distilled water.

3.22.5. Viewing

Ultrastructural analysis of the section was performed with Morgagni 268D with iTEM Imaging System. Specificity of labeling was assessed by the control test by incubating sections with rabbit pre-immune serum instead of the primary antibody.