Materials and Methods

3.1. Plant Material

Nursery grown mandarin (*Citrus reticulata*) seedlings, four month old obtained from Citrus Dieback Research Centre, Kalimpong [N 27° 12'457'' E 88° 14' .574''], Singbulli and Mirik Busty Area, Mirik [N 26° 52'.291'' E 88° 11' .174''] West Bengal, were used for experimental purposes. The selected seedlings initially maintained in 6" plastic pots and watered regularly for proper growth. After one year of growth seedlings were transferred in the earthenware pots (12" dia). These were kept in Glass House conditions (Plate 2,figsA-C) and after two years seedlings were planted in the experimental field. Suitable management practices were adopted in the field throughout the years.

3.2. Fungal culture

Fungal pathogen (*Macrophomina phaseolina*) was isolated from samples of diseased roots of mandarin (*Citrus reticulata*) plants grown in Mirik busty by culturing pieces of internal tissues. Infected root tissues were thoroughly washed in sterile water, treated with 0.1% $HgCl_2$ for 2-3 minutes, rewashed with sterile distilled water, transferred to potato dextrose agar (PDA) slants and incubated at 28°C for two weeks. The isolated organism was examined under microscope. Healthy seedlings of mandarin (*Citrus reticulata*) plants (1-year-old) were further inoculated with this isolated organism and incubated for a period of 4 weeks for completion of Koch's postulate. Subsequently, the infected roots were collected, washed, cut into small pieces, treated with 0.1% $HgCl_2$ for 2-3 minutes, rewashed with sterile distilled water, transferred to PDA slants and incubated at 28°C. At the end of two weeks, the reisolated organism was examined, compared with the original stock culture and its identity was confirmed following microscopic observations as *Macrophomina phaseolina.* Mycelia – septate, branched, hyaline when young becoming brown with age. Advancing zone of mycelia mat even and appressed. Sclerotia – black, moderate size (34-78 u in diameter), round or irregular (Plate 7 fig. D) uniformly reticulate with no difference in internal structure. The culture was maintained on PDA slants and stored under three different conditions [5°C , 20°C and 30°C (room temperature)]

Plate 2 (figs. A-C): Seedlings of *Citrus reticulata* maintained in nursery [A & B] and in glass house [C].

in sterile liquid paraffin. The culture was examined at a regular interval to test its viability and pathogenicity of the fungus.

Three other root pathogens- *Fusarium solani, Fusarium oxysporum and Fusarium graminearum* were obtained from Immuno-Phytopathology Laboratory, Department of Botany, N.B.U. which were also maintained with regular sub culturing in PDA for subsequent tests.

3.3. Isolation of microorganisms from Mandarin rhizosphere

Isolation of microorganisms from the rhizosphere of *Citrus reticulata* was carried out following, Warcup's soil plate method (1955) with a few modifications. The method favors isolation and enumeration of soil borne fungi, bacteria and actinomycetes. Five grams of soil particles loosely adhering to the roots were collected from six different locations of Darjeeling hills. The soil suspension was prepared by dissolving the soil sample in 30 ml sterile distilled water using magnetic stirrer for 1 h. The suspension was allowed to settle down till the two distinct layers were clearly visible. Then the upper light brown coloured layer was pipetted out and serial dilutions were made. One ml each of 10^{-3} and 10^{-4} dilutions were used for isolation by dilution plate technique (Kobayashi *et. al*, 2000) using Nutrient Agar (NA), King's B media, Potato dextrose agar (PDA) as well as *Trichoderma* selective media (TSM) as the growth media. The petriplates were then placed in an incubator for observation of the microbial growth after 24, 48 and 96h of incubation.

3.4. Isolation and Identification of AM spores from rhizosphere soil

Spores of arbuscular mycorrhizal fungi were isolated from rhizosphere soil of mandarin 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 and 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 -15°C to -20 °C 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).

3.5. Screening of root for mycorrhizal infection

Young roots from mandarin plants were dug out manually. The root sample was washed with tap water gently to free them from soil particles and stored in FAA (formaline aceto alcohol) prior to staining. For staining , root segment of 1cm each was put into the test tube and boiled in 10% KOH solution for 15-20 minutes on a water bath (sometimes even 60 minutes for hard roots), washed in tap water and stained with chlorazol black E (Beverage, 1970; Phillips and Hayman, 1970). For confirmation of infection, the presence of intercellular hyphae, vesicles and arbuscules or both characteristics was taken into consideration. Percent root colonization was determined following the method of Giovanetti and Mosse (1980).

3.6. Inoculation techniques and disease assessment

The inoculum of pathogen *M. phoaseolina* was prepared for inoculation of healthy citrus plants in sand maize meal medium supplemented with citrus root pieces, as it increases the survival capacity and viability of the pathogen in the soil. Initially, *M. phaseolina* was grown on PDA in Erlenmeyer flasks (250ml) for 2 days. Subsequently 30 sterilized mandarin root pieces (one inch long) were transferred to each flask and incubated for 15 days. Sand maize meal medium (50g) containing five such pieces covered with the mycelia and sclerotia were inserted in the rhizosphere of each plant. The inoculated plants were examined at an interval of 7 days up to a period of 28 days. Each time, the plants were uprooted, washed and symptoms noted. Finally roots were dried at 60^0C for 96h and weighed. Root rot index was calculated on the basis of percentage root area affected and they were graded into 6 groups and a value was assigned to each group (viz. no. root rot = 0; upto 10% root area affected =

0.10; 11-25% = 0.25; 26-50% = 0.50; 51-75% = 0.75; 76-100% = 1.0). The root rot index in each case was the quotient of the total values of the replicate roots and the number of roots (i.e. number of plants).

3.7. Assessment of mycelial growth.

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

3.7.1. 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 30°C. 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. Richards agar (RA):

KNO_3 - 1.00g, KH_2PO_4 - 50g, $MgSO_4$. $7H_2$ O - 0.25g, $FeCl_3$ - 0.002g, Sucrose - 3.00g, Agar - 2.00g, Distilled H_20 – 100ml

C. Carrot juice agar (CJA):

Grated carrot - 20.00g, Agar - 2.00g, Distilled water - 100 ml

D. Czapek dox agar (CDA):

$NaNo_3$ - 0.20g, $KHPO_4$ - 0.10g, $MgSo_4.7H_20$ - 0.05g, KCl - 0.05g, $FeSo_4.7H_20$ - 0.05g, Sucrose - 3.00g, Agar - 3.00g, Distilled water - 100ml

E. Potato sucrose agar (PSA):

Peeled potato - 40.00g, Sucrose - 2.00g, Agar - 2.00g, Distilled water - 100ml.

F. Malt extract peptone agar (MPA):

Malt extract - 20.00g, Peptone - 1.00g, Dextrose - 20.00g, Agar -

20.00g, Distilled water - 1L.

3.7.2. 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 28°C. 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), Richards medium and Nutrient broth (NB) 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 60°C for 96 h, cooled in desiccators and weighed.

3.8. Biochemical tests of microorganisms

3.8.1. Gram reaction

Smears of test organisms prepared from 24h old culture (on nutrient agar slant) with sterile distilled water were made in the centre of clean grease-free slides. The smears were air dried, heat fixed with crystal violet (crystal violet – 2.0g, 95% alcohol- 20ml, ammonium oxalate 1% W/V, aqueous solution – 80ml) stain for 1 min, washed with tap water for 5 sec, flooded with Burke's iodine solution (Iodine 1.0g, KI- 2.0g, distilled water 100ml) and allowed to react for 1 min. Slides were washed for 5 sec in 95% ethanol which was poured drop by drop by holding the slides in slanting position till the smears were decolorised, and then it was rinsed with water and dried. The smears were finally counter stained with safranin (2.5 w/v safranin in 95% ethanol-10ml, distilled water -100ml) for at least thirty seconds, rinsed with water and dried. The gram character and morphological characters were determined under oil-immersion objectives.

3.8.2. Endospore stain

The bacterial smear was prepared. The fixed slide was flooded with the solution of malachite green and the slide was placed over boiling water bath for five minutes. After rinsing, the smear was counter stained with safranin.

3.8.3. Catalase

Bacterial culture (24 h old) was flooded with 0.5 ml 10% H_2O_2 solution and gas bubble production indicated the positive reaction.

3.8.4. Urea digestion

Streaks were made on the slants containing urea medium and incubated at 37° C for 2-7 days. The change in colour of the medium indicates the presence of urease.

3.8.5. Casein hydrolysis

The mild agar was streaked with the bacteria and was incubated at 37° C and observed for the clear zone around the streaks.

3.8.6. Starch hydrolysis

The bacteria were streaked on sterilized starch agar plate (NA + 0.1% soluble starch) and incubated for five days at 37° C. The plates were flooded with Lugol's iodine solution. The clear zone underneath and around the growth indicates the starch hydrolysis.

3.8.7. Indole test

10ml of Davis Mingoli's broth supplemented with 0.1% tryptophan was inoculated with the isolate and incubated anaerobically at 37° C for 7 days. The culture was layered carefully with 2 ml of Ehrlich- Bobme (P-dimethylaminobenzaldehyde 10g, concentrated HCL 100ml) reagent on the surface, allowed to stand for a few minutes and observed for the formation of a ring at the medium reagent interface indicating the production of indole.

3.8.8. Siderophore production

Production of siderophore was detected by standard method of Schwyn and Neiland (1987) using blue indicator chrome azurol S (CAS). The bacteria were spot inoculated at the center of the plate and incubated for 12-15 days. The change in the colour of the medium around the bacterial spot was an indication of siderophore production.

3.8.9.Chitinase production

Production of chitinase was detected by standard method of Hsu and Lockwood (1975). Colonies showing zones of clearance against the creamy background were regarded as chitinase-producing strains.

3.8.10. Cellulase Production

Cellulose overlay agar plates were streaked with the 24h old bacterial cultures and incubated at 37°C for 7 days. The plates were observed for any clearing zone around or underneath the growth.

3.8.11. Protease production

Protease activity was detected on 3% (wt/vol) powdered milk-agar plates according to Walsh *et al.* (1996).

3.8.12. H_2S production

Slants containing SIM agar was inoculated with the test bacteria and incubated for 48h at 37°C. Darkening along the line of the slants indicated the production of H_2S by the organisms.

3.9. *In vitro* testing of rhizosphere microorganisms for antagonism towards root pathogen

3.9.1. Fungi

3.9.1.1. Solid medium

The efficacy of individual fungal isolates, from mandarin rhizosphere was tested *in vitro* for inhibiting growth of the pathogen (*M. phaseolina*) 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 of the fungal culture was inoculated at the other half of the Petri plate. For each test, three replicate plates were used. The plates were incubated for 7 days (depending upon the growth of the pathogen) at 28°C 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.

3.9.2. Bacteria

3.9.2.1. Solid medium

The obtained bacterial isolates were evaluated against root pathogen- *Macrophomina phaseolina* 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 the plate, incubation was undertaken for 5-7 days at 28°±2°C 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 *M. phaseolina*, were selected for further evaluation and identification.

3.9.2.2. Liquid medium

To assess the possible antagonism between the root pathogen (*M. phaseolina*) and bacteria, NB was selected for growth of selected bacteria (which showed inhibition in solid medium). Agar block (4mm) containing 7 day old mycelia of the fungus and 0.5ml of bacterial suspension ($1x10^6$ cfu/ml) were used as inoculum for each flask (50 ml NB/250 ml flask). The mycelium grown without bacterial isolates in similar medium was taken as control. The cultures were incubated at 28 ±2°C and after 7 days of incubation mycelia were washed thoroughly with sterile distilled water to remove bacteria as far as possible and

harvested by staining through muslin cloth and mycelial dry weights were determined. Three replicates were taken in each case.

3.10. Scanning Electron Microscopic observation of selected beneficial microorganisms

Selected beneficial microorganisms (BCA and PGPR) were examined under scanning electron microscopy (SEM). Samples were prepared according to a modification of the method described by King and Brown (1983). Test isolates were grown on PDA plates for l0 days in daylight at room temperature. Small pieces of the agar (less than 1 cm), with aerial sporulating culture attached, were excised from each plate and transferred to the interior surface of a dry glass Petri dish lid. Efforts were made not to disturb the attached culture. Steps that involved exposing the samples to the atmosphere were performed quickly to minimize air-drying artifacts. The specimen dishes were then placed in vapour diffusion dehydration (VDD) assembly, and a vacuum was drawn as described by King and Brown (1983). All samples were left in the VDD assembly where a maximum level of dehydration was achieved. The vacuum was released slowly and the specimen dish was removed from the desiccator. Each sample was placed within separate aluminum "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 9.5-15 Kev.

3.11. Assessment of bacterial growth

For assessment of bacterial growth in liquid medium, 1ml of bacterial suspension was inoculated into the medium and allowed to grow for desired period. Following growth, absorbance was noted in a colorimeter at 600nm. Absorbance was converted into cfu/ml from a standard where known concentration of bacterial suspension was used. The cfu values were counted to log whenever needed. For assessment of bacterial growth, different media were used. These are as follows;

A. Nutrient broth (NB):

Peptone - 5.0g, Beef extract - 3.0g, NaCl - 5.0g, Yeast extract - 3.6g, Water – 1L, pH – 7.4 ± 0.2

B. Nutrient agar medium (NA):

Peptone - 5gm, NaCl - 5gm, Yeast extract - 1.5 gm, Beef extract - 1.5g, Agar - 20g, Water - 1L

C. Luria broth (LB):

Peptone – 10.0 g, NaCl - 5.0g, Yeast extract - 1.5 gm, Distilled H_20 - 1L, pH 7.2 ± 0.2

D. Nutrient sucrose broth (NSB):

Sucrose – 1.5g, Yeast extract - 1.2 gm, Peptone – 1.2.0 g, Beef extract - 0.6g, Distilled H_20 – 300 ml, pH – 7.2 ± 0.2

3.12. *In vitro* Screening and Evaluation of phosphate solubilizing activity of isolated microorganisms

3.12.1. Screening

Preliminary screening for phosphate solubilization was done by a plate assay method using Pikovskaya (PVK) agar medium supplemented with Tricalcium phosphate (TCP) and pH of the medium was adjusted to 7.0 before autoclaving. One gram soil sample was suspended in 9ml sterile distilled water in a tube for serial dilutions, and 1ml aliquots were transferred to PVK medium. The plates were incubated at 28±2°C for 7 days with continuous observation for colony diameter. Transparent (halo) zones of clearing around the colonies of microorganisms indicate phosphate solubilization and each colony was carefully transferred, identified and further used for quantitative determination of phosphate solubilization.

3.12.2. Evaluation

Evaluation of phosphate solublizing activity of fungal isolates was done by growing the isolates in the two sets of Pikovskaya's liquid medium amended with 0.5% tricalcium phosphate and 0.5 % rock phosphate separately over a period of 10 days at 28^{0}C with constant shaking at 100 rpm in a rotary incubator. Quantitative estimation of phosphate was done following amonium molybdate ascorbic acid method as described by Kundsen and Beegle (1988). Amount of phosphate utilized or solubilized by the isolates were expressed as mg/L phosphate utilized by deducting the amount of residual total phosphate from the initial amount of phosphate source added to the modified Pikovskaya's liquid medium (yeast extract, 0.50 g/L, dextrose, 10.0 g/L, calcium phosphate/rock phosphate, 5.0 g/L, ammonium phosphate, 0.50 g/L, potassium chloride, 0.20 g/L, magnesium sulphate, 0.10 g/L, manganese sulphate, 0.0001g/L, ferrous sulphate, 0.0001 g/L, pH, 6.5)amended with 0.5 % tricalcium

phosphate and 0.5 % rock phosphate. Liquid medium (50 ml) was inoculated with 5 % v/v of the spore suspension prepared from the 7 days old culture grown on PDA slants and incubated at room temperature for 4 days with routine shaking at 100 rpm. The initial pH of the medium was recorded. The mycelia were harvested after 10 days of incubation by filtering and the change in the pH of the culture filtrate was recorded after centrifuging the medium at 5000 x g for 5 min. on a table centrifuge.

Phosphate measurement in the soil was estimated by ammonium molybdate-ascorbic acid method where, 2ml of the aliquot of the soil extract was mixed with 8ml of the colorimeter working solution containing 60 g/L ammonium paramolybdate, 1.455 g antimony potassium tartarate, 700ml/L conc. sulfuric acid and 132 ml/L of ascorbic acid and mixed thoroughly and incubated for 20 min. till the colour developed. Percent transmittance of the solution was taken on a colorimeter with the wavelength set at 882 nm.

3.13. Application of PGPR

3.13.1. Soil drench

The bacteria were grown in NB for 48 h at 28°C 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 seedling from sleeves. Applications were done @ 0f 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.

3.13.2. Foliar spray

The bacterial pellet suspended in sterile distilled water at a concentration of $3x10^6$ 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. The growth parameters such as number of leaves, branches and height were observed.

3.14. Inocula preparation and application of biocontrol fungi

3.14.1. AMF. Spores of *Glomus mosseae* were separated from the mass of other AM spores by fine tweezers and needles under dissecting microscope and were washed by distilled water

several times to remove the adhered debris followed by inoculation in the roots (7-10 days old) of *Sorghum bicolor*, *Cynodon dactylon*, and *Zea mays* grown in black plastic pots (12inch) having autoclaved soil to discard the presence of other fungal propagules. After 45 days the presence of spores of *G. mosseae* were verified and inocula were prepared by mixing the chopped roots of sorghum plants with the potted soil where extra radical spores of *G. mosseae* were present. Approximately > 175 spores / 100gms could be considered as potent inocula for application.

3.14.2. BCA. Inoculum of *Trichoderma asperellum* was prepared by inoculating wheat bran (sterilized) with 5 mm disc of the fungus and incubating at 28 °C for 10 days. To each pot containing either *F. solani* infested or control soil (2000 g), 10 g of the wheat bran colonized by *T. asperellum* was mixed to give a concentration of 10^5 cfu / g of soil as described by Chakraborty *et al.* (2003).

3.15. Extraction and estimation of soluble proteins

3.15.1. 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± 1°C. 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 4°C and then centrifuged at 10.000rpm for 30 min, at 4°C to eliminate cell debris. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring in ice bath and kept overnight at 4°C. After this period, the mixture was centrifuged (10,000rpm) for 30 minute at 4° 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 0.005 M sodium phosphate buffer (pH 7.2) with six changes. The dialysate was stored at -20°C and used as antigen fro the preparation of antiserum and other experime

3.15.2. Root

Soluble protein was extracted from mandarin roots following the method of Chakraborty *et al.*, (1995). Root tissues were frozen in liquid nitrogen and ground in 0.05 mM sodium

phosphate buffer (pH 7.2) containing 10 mM $Na_2 S_2 O_5$, 0.5 mM $MgCl_2$ and 2mM PMSF was added during crushing and centrifuged at 4°C for 20 min at 12000rpm. The supernatant was used as crude protein extract.

3.15.3. Estimation of 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 and 1ml of 2% sodium potassium tartarate, added to 100ml of 2% $Na_2 CO_3$ in 0.1 NaOH) was added. This was incubated for 15 min at room temperature and then 0.5ml of 1N Folin Ciocalteau reagent was added and again incubated for further 15 min following which optical density was measured at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

3.16. 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

3.16.1. Preparation of stock solution

Following stock solution were prepared

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

A 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 4°C 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 4°C 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 4°C 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.

3.16.2. 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	0.5
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.

3.16.3. 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.

3.16.4. 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.

3.16.5. 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 °C 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 °C with constant shaking until the background become clear

3.17. Immunological studies

3.17.1. Preparation of antigen

3.17.1.1. 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 °C and then centrifuged at 10.000rpm for 30 min at 4 °C to eliminate cell debris. The supernatant was collected and stored in -20 °C and used as antigen for the preparation of antiserum.

3.17.1.2. Root antigen

Root antigen was extracted from mandarin roots following the method of Chakraborty *et al.*, (1995). Root tissues were frozen in liquid nitrogen and ground in 0.05 mM sodium phosphate buffer (pH 7.2) containing 10 mM $Na_2 S_2 O_5$, 0.5 mM $MgCl_2$ and 2mM PMSF was added during crushing and centrifuged at 4°C for 20 min at 12000 rpm. The clear supernatant was used as antigen.

3.17.2. Raising of polyclonal antibodies

3.17.2.1. 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.

3.17.2.2. 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.

3.17.2.3. 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 60°, 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 37°C 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 -20°C as crude antisera. The serum was used for double diffusion analysis, dot blots analysis and Enzyme Linked Immunosorbent Assay (ELISA)

3.17.3. Purification of IgG

3.17.3.1 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_4 SO_4) ammonium sulphate was taken and pH adjusted to 6.8, stirring the mixture for 16h at 20° C in magnetic stirrer. The precipitate thus formed was collected by centrifugation at 12,000 rpm for 1h at 22 ° C for 1 h. Supernatant was discarded and pellet was used for further steps.

3.17.3.2 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.

3.17.3.3 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).

3.17.4. Immunological assays

3.17.4.1. Agar gel double diffusion

3.17.4.1.1. 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 ° 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.

3.17.4.1.2. 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 25°C for 72h. Precipitation reaction was observed in the agar gel only in cases where common antigen was present.

3.17.4.1.3. 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 50° C

3.17.4.2. 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 °C 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 °C 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 4°C 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 °C 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, ThermoLabsystems) at 405 nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.17.4.3. 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 °C 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 37°C. 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.

3.17.4.4. Western blot analysis

Protein samples were electrophoresed on 10% SDS-PAGE gels as suggested by Laemmli (1970) and electrotransferred to NCM using semi-dry Trans-blot unit (BioRad) and probed with PAbs of the pathogen (*M. phaseolina*) 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.

3.17.4.5. 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)

3.17.4.5.1. Fungal mycelia

Fungal mycelia were grown in liquid Richards's 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.

3.17.4.5.2. Cross section of mandarin 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 section were observed using Leica Leitz Biomed Microscope with fluorescence optics equipped with UV- filter set I-3 and photograph was taken.

3.18. Isolation of genomic DNA

Isolation of fungal genomic DNA was done by growing the fungi for 3-4 days. For bacteria, the growth was taken for 24 hr. Liquid nitrogen was used for crushing the cell mass for both cases.

3.18.1. Preparation of genomic DNA extraction buffer

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

Lysis Buffer
50 mM Tris, pH 8.0
100 mM EDTA
100mM NaCl
1% SDS

Genomic DNA Buffer
10 mM Tris, pH 8.0
0.1 mM EDTA

CTAB Buffer
2% CTAB
1.5% PVP K 30
1.4 mM Nacl
20 mM EDTA
100mM Tris HCL pH 8.0
0.1% B-mercaptoethanol

3.18.2. Genomic DNA extraction

Isolation of fungal genomic DNA was done by growing the fungi for 3-4 days. The mycelia were incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH8.0), 100 mM NaCl and 2% SDS, for 1 hr at 60^0C followed by centrifugation at 12,000 rpm for 15 min., whereas genomic DNA was extracted from isolates of bacteria and actinomycetes using CTAB buffer. The supernatant was then extracted with equal volume of water saturated phenol and further centrifuged at 12,000 rpm for 10 min; the aqueous phase was further extracted with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 12,000 rpm for 15

min; the aqueous phase was then transferred in a fresh tube and the DNA was precipitated with chilled ethanol (100%). DNA was pelleted by centrifuging at 12000 rpm for 15 min, washed in 70% ethanol and air dried.

3.18.3. 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°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.

3.18.4. Measurement of DNA concentration using Spectrophotometry

The pure sample was (without significant amounts of contaminants such as a proteins, phenol, agarose, or other nucleic acids), used to quantify DNA. For quantitating DNA absorbance at wavelengths of 260 nm and 280 nm were taken. Quantification was done as follows:

1 O.D. at 260 nm for double-stranded DNA = 50 ng/ul of dsDNA

1 O.D. at 260 nm for single-stranded DNA = 20-33 ng/ul of ssDNA

Pure preparations of DNA have OD_{260}/OD_{280} value 1.8. If there is contamination with protein or phenol, this ratio will be significantly less than the value given above, and accurate quantitation of the amount of nucleic acid will not be possible.

3.18.5. 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.

3.18.5.1. 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.

3.18.5.2. 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.

3.19. RAPD PCR analysis

For RAPD, random primers were selected (Table-1). PCR was programmed with an initial denaturing at 94°C for 4 min. followed by 35cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 70°C for 90 s and the final extension at 72°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 by horizontal electrophoresis.

3.19.1. RAPD primers

The following primers were used for RAPD analysis in the study:

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC
RAPD primers				
AA-04	CAGGCCCTTC	10	38.2	70%
OPA-4	AATCGGGCTG	10	39.3	60%
A-11	AGGGGTCTTG	10	31.8	76%
A-5	AGGGGTCTTG	10	36,8	73%
OPD6	GGGGTCTTGA	10	32.8	83%
OPA1	CAGGCCCTTC	10	38.2	70%

3.19.2. Amplification conditions

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

3.19.3. Analysis of RAPD bands

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

3.19.4. 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.

3.19.5. 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.

3.19.6. 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 *In Silico* 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.

3.20. ITS PCR analysis

All isolates of *Trichoderma* 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°C for 5 min. followed by 30 cycles of denaturation at 94° C for 30 sec, annealing at 59°C for 30 sec and extension at 70°C for 2 min and the final extension at 72 °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.

3.20.1. ITS- PCR primers

The following primers were used to amplify ITS regions:

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC	Amplicaon size (bp)	References
Macrophomina sp.						
ITS 1	TCCGTAGGTGAACCTGCG	18	61	56%	~620	White *et al.* (1990)
ITS4	TCCTCCGCTTATTTGATATGC	21	63	59%		
Fusarium sp.						
Fcg17F	TCGATATACCGTGCGATTTCC	21	65	47%	~570	Nicholson *et al.* (1998)
Fcg17R	TACAGACACCGTCAGGGGG	19	66	63%		
Trichoderma sp.						
T/ITS 1	TCTGTAGGTGAACCTGCGG	19	63.9	57%	~600	White *et al.*, (1990)
T/ITS4	TCCTCCGCTTATTGATATGC	20	61.5	45%		

3.20.2. Amplification conditions

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

3.20.3. Sequencing of rDNA gene

The rDNA was used for sequencing purpose. DNA sequencing was done bi-directionally using the ITS primer pairs by Genei Bangalore.

3.20.4. Sequence analysis

DNA sequence information was analyzed using bioinformatic algorithms tools e.g. Bioedit, MEGA 4, NTSYSpc as well as the few online softwares.

3.20.5. Chomatogram of sequence

The chromatogram of the DNA sequence was analysed by the software Chromus.

3.20.6. Editing and alignment of sequence data

All the DNA sequences edited by using the software BioEdit and aligned with Clustral W algorithms.

3.20.7. 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.

3.20.8. 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.

3.21. Denaturing Gradient Gel Electrophoresis (DGGE)

3.21.1. Materials

(A) 40% Acrylamide:bisacryl-amide (37.5:1)

(B) 50 x DGGE/TAE buffer solution

Trizma-Base:	484.4 grams
Sodium-Acetate:	272.0 grams
trisodium EDTA	37.2 grams
H_2O	2 liters

pH 7.40 adjusted with about 230 ml of glacial acetic acid.

(C) Preparation of Denaturants

100% Denaturant

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

Filled up to 100 ml with distilled H_2O.

0% Denaturant

38.5% Acrylamide	16.9 ml
50x DGGE/TAE	2.0 ml
Ammonium Persulphate	10% (w/v)
TEMED	20µl

3.21.2. Methods

3.21.2.1. 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 again with 96% ethanol. Both 1mm spacers were also cleaned with

96% ethanol and placed on the large glass plates. The clamps were screwed 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 was checked. Then the sandwich was placed on top of the rubber gasket and the handles pressed down.

3.21.2.2. Preparing 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 following table.

UF solution	[UF](%)	[Acrylamid/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 low and high solutions according to 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.

3.21.2.3. Running a gel

Fresh 0.5x TAE buffer was added to the buffer tank to the mark "Fill". The DCode™ Universal Mutation Detection System (Bio-Rad) was switched on at least 60 min 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™ was then switched off and the lid taken off after 1 minute. The sandwich holder was slided into the buffer tank, with the red dot of the cathode at the right side. The DCode™ pump and the stirrer underneath the tank were switched on (300 rpm) until samples were loaded.

3.21.2.4. Staining of gels and photography

Before taking DGGE units out from the tank, the run - evaporated H_2O was replaced up to the marked level. Carefully the DGGE unit was dismantled. The ethidium bromide stain was added into a tray with 50XTAE buffer and the gel was stained for 5 minutes and distained with running buffer. The gel was photographed under UV transilluminator.

3.22. Extraction and Assay of defense enzyme activity

3.22.1. 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 °C using mortar and pestle. The extract was then centrifuged at 10000 rpm for 15 min at 4 °C 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 40°C for 10minutes. The reaction was stopped by adding 375µl dinitrosalicylic reagent and heating for 5 min on 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.

3.22.2. 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 (pH5). 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 °C 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 phosphate buffer (1M) pH7.1 and incubated with 20µl of (3%w/v) desalted snail gut enzyme Helicase (Sigma) for 1hour.

After 1h, the pH of the reaction mixture was brought to 8.9 by 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-dimethylaminobenzaldehyde) reagent. The mixture was incubated for 20 min at 37 °C.

Immediately therefore absorbance value at 585nm was measured using a UV-VIS spectrophotometer. N-acetyl glucosamine (GlcNAc) was used as standard. The enzyme activity was expressed as µg GLcNAc min^{-1} mg^{-1} fresh tissues.

3.22.3. 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 Centrifuge in 15000 rpm for 20 minutes at 4°C. Supernatant was collected and after recording its volume, was immediately used for assay or stored at-20°C.

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 30 µM L- phenylalanine and 0.5ml of supernatant in a total volume of 3ml. Following incubation for 1 h at 40 °C 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.

3.22.4. 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 °C. after centrifugation the supernatant was collected and after recording its volume was immediately used for assay or stored at -20 °C (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 H_2O_2, 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 *et al.*, 1993). Specific activity was expressed as the increase in absorbance at 460 nm g^{-1} tissue/ min^{-1}.