3.1. Plant Material

Nursery grown mandarin (*Citrus reticulata*) seedlings, two month old obtained from IARI Kalimpong, Nirmaldass Orchard Gurung Brothers Nursery Baramangwa Busty, Darjeeling, Bijanbari, and Mirik were used for experimental purposes. One year old *C. limonia* seedlings were obtained from CDRS, Kalimpong and *C. medica* from Padmaja park, University of North Bengal. 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 and after two years seedlings were planted in the experimental field. Suitable management practices were adopted in the field throughout the years (Fig 3).

3.2. 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.
Fig 3: Maintenance of mandarin saplings in glass house
3.2.1. Isolation of AMF

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.

3.2.2. Isolation of bacteria and fungi

Isolation of microorganisms from the rhizosphere of Citrus reticulata was carried out following, Warcup’s soil plate method (1955) with a few modifications. Five grams of soil particles loosely adhering to the roots were collected from from healthy mandarin plants of different geographical locations of hills and plains. 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.
3.2.3. Isolation of Mycorrhiza Helper Bacteria

Mycorrhiza Helper Bacteria (MHB) were isolated as per the protocol of Budi et al. (1999) with a few modifications. Spores of *Gi gigantea* (45-50) were sonicated at 25 Hz for two minutes to remove the debris adhered to the spores. They were then washed 3-4 times gently in sterile water to remove loosely adhering particles and transferred to fresh sterile water, vortexed again and rinsed with sterile water until this remained clear. They were then transferred using a sterile Pasteur pipette to a sterile Millipore filtering apparatus (Millipore SA) with a 0.8- mm filter. After a rinse with 50 ml sterile water, the spores were washed successively with three sterilizing solutions: (1) 96% ethanol, (2) a mixture of 2% Chloramine T (w/v), 0.02% streptomycin (w/v), 0.01% gentamycin (w/v) and two drops of Tween 20, (3) 6% calcium (Ca) hypochlorite (w/v), and were plated in NA medium aseptically under laminar air flow. Total number of twelve set ups were prepared for the isolation of MHB.

3.3. Histopathological studies of mandarin 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).

3.4. Morphological characterization of AMF spores

3.4.1. Microscopical

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 CaCl₂ in 1 L of boiled distilled water) at -15°C 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).

3.4.2. SEM

Dominant mycorrhizal fungal spores were examined under scanning electron microscopy (SEM). Selected AMF 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 9.5-15 Kev.

3.5 Biochemical tests of microorganisms

3.5.1. Screening for phosphate solubilization

Primary phosphate solubilizing activities of bacterial isolates were carried out by allowing the bacteria to grow in selective medium i.e., Pikovskaya’s agar (Himedia-M520; ingredients- yeast extract-0.50 g/l, dextrose- 10.00 g/l, calcium phosphate- 5.00 g/l, ammonium sulphate- 0.50 g/l, potassium chloride- 0.20 g/l, magnesium sulphate-0.10 g/l, manganese sulphate- 0.0001 g/l, ferrous sulphate- 0.0001 g/l and agar- 15.00 g/l) for 7 to 10 days at 37°C (Pikovskaya, 1948). The appearance of transparent halo zone around the bacterial colony indicated the phosphate solubilizing activity of the bacteria.

3.5.2. Siderophore production

The bacterial isolates were characterized for siderophore production following the method of Schwyn and Neiland (1987) using blue indicator dye, chrome azurol S (CAS).
For preparing CAS agar, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1 mM FeCl3.6H2O in 10 mM HCl) and volume made up to 1L. With constant stirring this solution was added to 72.9 mg hexa-decytrimethyl ammonium bromide (HDTMA), dissolved in 40 ml water. The resultant dark blue liquid was autoclaved. The dye solution was mixed into the medium along the glass wall with enough agitation to achieve mixing without the generation of foam, and poured into sterile petriplates (20 ml per plate). The plates were inoculated with the bacteria and incubated for 10-15 days till any change in the color of the medium was observed.

3.5.3. HCN production

Production of hydrocyanic acid was determined using the procedure described by Reddy et al. (1991) with slight modification. The selected bacterial isolates were grown at room temperature (37°C) on a rotary shaker in nutrient broth (NB) media. Filter paper (Whatman no.1) was cut into uniform strips of 10 cm long and 0.5 cm wide saturated with alkaline picrate solution and placed inside the conical flasks in a hanging position. After incubation at 37°C for 48 hr, the sodium picrate present in the filter paper was reduced to reddish compound in proportion to the amount of hydrocyanic acid evolved. The color was eluted by placing the filter paper in a clean test tube containing 10 ml distilled water and the absorbance was measured at 625 nm.

3.5.4. IAA production

For detection and quantification of IAA, the selected bacterial cells were grown for 24 h to 48 h in high C/N ratio medium. Tryptophane (0.1 mM) was added in order to enhance acetic acid (IAA) production by the bacteria (Prinsen et al. 1993). Production of IAA in culture supernatant was assayed by Pillet-Chollet method as described by Dobbelaere et al. (1999). For the reaction, 1 ml of reagent, consisting of 12 g FeCl₃ per litre in 7.9 M H₂SO₄ was added to 1 ml of sample supernatant, mixed well, and kept in the dark for 30 min at room temperature. Absorbance was measured at 530 nm.

3.5.5. 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.

3.5.6. Catalase activity

Bacterial culture (24 hour old) was flooded with 0.5 ml 10% H$_2$O$_2$ solution and gas bubbles production indicated the positive reaction.

3.5.7. Protease production

Protease activity was detected on 3% (wt/vol) powdered milk-agar plates according to Walsh et al. (1995).

3.5.8. H$_2$S Production

Slants containing SIM agar inoculated with the test bacteria and incubated for 48h at 37ºC. Darkening along the line of the slants indicated the production of H$_2$S by the organisms.

3.5.9. Urease production

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

3.6. Fungal pathogen

3.6.1. 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.

3.6.2.1. 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. Czapek dox agar (CDA):
   NaNO₃ - 0.20g, KHPO₄ - 0.10g, MgSO₄.7H₂O - 0.05g, KCl - 0.05g, FeSO₄.7H₂O - 0.05g, Sucrose - 3.00g, Agar - 3.00g, Distilled water - 100ml

C. Potato sucrose agar (PSA):
   Peeled potato - 40.00g, Sucrose - 2.00g, Agar - 2.00g, Distilled water - 100ml.

### 3.6.2.2. 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.

### 3.7. In vitro screening and evaluation of phosphate solubilizing activity of isolated microorganisms

#### 3.7.1. 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±2ºC 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.
3.7.2. 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°C with constant shaking at 100 rpm in a rotary incubator. Quantitative estimation of phosphate was done following ammonium 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.

3.8. Antifungal tests against pathogens in Dual Plate Culture

3.8.1. Antifungal test of PGPR

The obtained bacterial isolates were evaluated against root pathogens- *F. solani* and *F. oxysporum* 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°C±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 *F. solani* and *F. oxysporum*, were selected for further evaluation and identification.
3.8.2. Antifungal test of BCA

The efficacy of BCA(Trichoderma sp.) isolated from mandarin rhizosphere was tested in vitro for inhibiting growth of the pathogen (F. solani and F. oxysporum) in dual culture using PDA. Each fungal isolate was placed at one side of the agar plate about 1 cm away from the edge and a 4 mm 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 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. Mass multiplication of microorganism and their application

3.9.1. Mass multiplication of AMF

Three hosts were selected for mass multiplication of AMF spores (maize, sorghum and turf grass). Spores of C. reticulata were multiplied in all three hosts but spores of C. medica and C. limonia were multiplied in maize only. AMF spores were isolated from rhizosphere of C. reticulata, C. medica and C. limonia as described. 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 (12 inch) 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 were 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 / 100 gms could be considered as potent inocula for application.

3.9.1.1. Single cell-line culture

In order to develop pure cell line culture of two specific AM fungi (Glomus fasciculatum and Gigaspora gigantea) following experimental set up were made using sterilized pre soaked sorghum seedlings. Sorghum seeds were kept in the plate over a wet filter paper and it was covered with black paper to avoid light. Within 96h seeds germinate and shoots came out from the open cut end. Selected AMF spore of G. fasciculatum and Gi. gigantea were carefully inoculated on the root surface and closed by black paper again.
Then 4-5 days after inoculation the small seedlings were transferred in pot having sterile soil-sand mixture (50: 50). After 90 days the desired spore of a single species were harvested. After harvesting the same process is repeated thrice to get the desired spore and to discard other entities. Once pure line culture is obtained, the same experiment was setup for mandarin seedlings. The inoculated seedlings were then maintained in glass house in sterilized soil (Fig. 4).

Fig 4: Single spore inoculation of AMF in mandarin roots. Experimental setup (A-F); mass of *Gigaspora* spore (G); Single *Gigaspora* spore (H); Maintenance of inoculated seedlings in sterilized soil (I-L).
3.9.2. Mass multiplication of BCA

3.9.2.1. Wheat bran culture

Inoculum of *Trichoderma asperellum* and *Trichoderma harzianum* were prepared by inoculating wheat bran (sterilized) with 5 mm disc of the fungus and incubating at 28 ºC 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 $10^5$ cfu / g of soil as described by Chakraborty *et al.* (2003).

3.9.2.2. 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 (Fig 5).

![Tricho compost preparation](image)

**Fig. 5** Tricho compost preparation. Pile of compost materials (A), Turning of compost (B), harvested Tricho-compost in sacks (C)
3.9.3. Mass multiplication of PGPR

3.9.3.1. Soil drench

The bacteria were grown in NB for 48 h at 28ºC and centrifuged at 12,000 rpm for 15 minutes. 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 $3 \times 10^6$ cfu ml$^{-1}$. The bacterial suspension was applied to the pots during transplantation of seedlings. Applications were done at an 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.

3.9.3.2. Foliar spray

The bacterial pellet suspended in sterile distilled water at a concentration of $3 \times 10^6$ 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.

3.9.3.3. 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 $3 \times 10^9$ 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 ($12 \times 10^{10}$ bacterial cells).

3.9.4. Preparation of inoculum of fungal pathogen

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

3.10. Growth promotion studies following application of AMF and PGPR

3.10.1. Assessment of plant growth

Plant growth promotion was assessed by examining the plant at intervals of 1 month up to a period of 4 months. The growth parameters such as number of leaves, branches and height were observed.

3.10.2. Assessment of soil phosphate mobilization

3.10.2.1. Extraction of Soil phosphate

Soil sample (1g) was air dried and suspended in 25 ml of the extracting solution (0.025N H$_2$SO$_4$, 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).

3.10.2.2. 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.

3.11. In vivo assessment of disease

3.11.1. Assessment of root rot index

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°C 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).

3.11.2. Assessment of defense enzymes in leaves and roots

3.11.2.1. β-1, 3-glucanase (E.C. 3.2.3.39)

Estimation of β-1, 3-glucanase activity was done by following the laminarin dinitrosalicylate method described by Pan et al. (1991). The crude enzyme extract of 62.5 µl was added to 62.5 µl of laminarin (4 %) and then incubated at 40°C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylic reagent and heating for 5 min on a boiling water bath. The resulting colored solution was diluted with 4.5 ml of water, vortexed and absorbance was recorded at 500 nm. 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 tissue.

3.11.2.2. Chitinase (E.C. 3.2.1.14)

Chitinase activity was measured according to the method described by Boller and Mauch (1988). Assay mixture consisted of 10µl of 1M Na-acetate buffer (pH4), 0.4 ml enzyme solution and 0.1 ml colloidal chitin. Colloidal chitin was prepared as per the method of Roberts and Selitrennikoff (1988). Incubation was done for 2 hrs at 37°C and centrifuged at 10,000 r.p.m for 3 min. 0.3 ml supernatant, 30µl of 1M K-PO₄ buffer (pH7.1) and 20µl Helicase (3%) were mixed and allowed to incubate for 1 h at 37°C. 70µl of 1M Na-borate buffer (pH9.8) was added to the reaction mixture. The mixture was again incubated in a boiling water bath for 3 min and rapidly cooled in ice water bath. 2 ml DMAB (2% di methyl amino benzaldehyde in 20% HCl) was finally added and incubated for 20 min at 37°C. The amount of GlcNAc released was measured spectrophotometrically at 585 nm using a standard curve and activity expressed as μg GlcNAc released /min/ g fresh wt. tissue.

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

Enzyme was extracted by the method described by Chakraborty et al. (1993). Leaf samples were crushed in liquid nitrogen and extracted using 5 ml of sodium borate buffer (pH8.8) containing 2 mM β mercaptoethanol in ice followed by centrifugation at 15000 rpm for 20 min at 4°C. The supernatant was collected and after recording its volume, used immediately for assay or stored -20°C.
3.11.2.4. Peroxidase (E.C. 1.11.1.7)

For the extraction of peroxidase 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 β-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).

3.11.2.5. Extraction and estimation of phenols

3.11.2.5.1. 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.

3.11.2.5.2. Estimation

3.11.2.5.2.1. 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.

3.11.2.5.2.2. O-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 (NaNO₂ - 10 g, Na₂MoO₄ - 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
Quantity of the O-dihydroxy phenol was estimated using caffeic acid as standard.

3.12. 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.

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.

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. 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.

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.

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 % H₂O₂ (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.

3.13. Extraction and estimation of soluble proteins

3.13.1. 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 from the preparation of antiserum and other experiment.
3.13.2. Leaf

Soluble protein was extracted from mandarin leaves following the method of Chakraborty et al., (1995). Leaf tissues were frozen in liquid nitrogen and ground in 0.05 mM sodium phosphate buffer (pH 7.2) containing 10 mM Na₂S₂O₅, 0.5 mM MgCl₂ and 2mM PVP was added during crushing and centrifuged at 4°C for 20 min at 12000rpm. The supernatant was used as crude protein extract.

3.13.3. 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 Na₂S₂O₅, 0.5 mM MgCl₂ and 2mM PVP was added during crushing and centrifuged at 4°C for 20 min at 12000rpm. The supernatant was used as crude protein extract.

3.13.4. 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% Na₂CO₃ 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).

3.14. 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

3.14.1. 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 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 β- 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 β- mercaptoethanol, 2 ml of 10% SDS, 10 mg bromophenol blue, 1ml glycerol in 6.8 ml of distilled water.

**3.14.2. 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.

<table>
<thead>
<tr>
<th>Name of the compound</th>
<th>10% Resolving gel (ml)</th>
<th>5% Stacking gel (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>2.85</td>
<td>2.10</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>2.55</td>
<td>0.5</td>
</tr>
<tr>
<td>Tris*</td>
<td>1.95</td>
<td>0.38</td>
</tr>
<tr>
<td>10%SDS</td>
<td>0.075</td>
<td>0.030</td>
</tr>
<tr>
<td>10%APS</td>
<td>0.075</td>
<td>0.030</td>
</tr>
<tr>
<td>TEMED**</td>
<td>0.003</td>
<td>0.003</td>
</tr>
</tbody>
</table>

*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.

### 3.14.3. 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; Bovine 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.
3.14.4. 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.

3.14.5. 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.

3.15. Immunological studies

3.15.1. Preparation of antigen

3.15.1.1. 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 MgCl₂ 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.

3.15.1.2. 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 Na₂ S₂ O₅, 0.5 mM
MgCl₂ and 2mM PVP was added during crushing and centrifuged at 4°C for 20 min at 12000 rpm. The clear supernatant was used as antigen.

3.15.1.3. AMF antigen

Spores of *G. mosseae* and *G. gigantea* were isolated from rhizosphere soil of mandarin by wet sieving and decanting method as described before. With the help of a dissecting microscope parasitized spores, plant debris etc were separated and clean spores of *G. mosseae* and *G. gigantea* were isolated. 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.

3.16.2. Raising of polyclonal antibodies

3.16.2.1. 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.

3.16.2.2. 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.

3.16.2.3. 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 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).

3.16.3. Purification of IgG

3.16.3.1 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 (NH₄ SO₄ ) 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.

3.16.3.2 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.

3.16.3.3 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).

3.16.4. Immunological assays

3.16.4.1. Agar gel double diffusion

3.16.4.1.1. 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 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.

3.16.4.1.2. Diffusion

Agar gel double diffusion tests were carried out using antigen and antisera 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.

3.16.4.1.3. 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% NaN₃) 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.

3.16.4.2. 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 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 antiserum 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, ThermoLabsystems) at 405 nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.16.4.3. 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 MgCl₂
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.

3.16.4.4. 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 (F. solani and F. oxysporum) 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.

3.16.4.5. 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). 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.
3.16.4.5.1. 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.

3.16.4.5.2. 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 antisera 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. Fluorescence 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.

3.16.4.5.3. AMF in mandarin root

Roots of maize plants (4 months) and mandarin plants (2 years old) in which AMF spores of mandarin 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. Fluorescence 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.

3.16.4.5.4. AMF spores

*Glomus* and *Gigaspora* spores along with hyphae were carefully separated from the root and washed. Selected spores were taken in grooved slides and left overnight in antibacterial solution. They were washed thrice with PBS pH 7.2. Antigen was given separately with a dilution of 1:40 and left overnight. The next day the spores were again washed thrice with PBS-Tween. FITC and RITC were given separately at a dilution of 1:10 and again left overnight. Finally, the next day, spores were mounted in grease free slides in 10% glycerol and observed under Leica Leitz Biomed Microscope with fluorescence optics equipped with UV- filter set I-3 and photograph was taken.

3.17. 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.

3.17.1. 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
- 100 mM 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

3.17.2. 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°C 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.

3.17.3. 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.

3.17.4. 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 \( \text{OD}_{260}/\text{OD}_{280} \) 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.

### 3.17.5. Agarose gel electrophoresis 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.

#### 3.17.5.1. 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.

#### 3.17.5.2. 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.

### 3.18. 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 35 cycles 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.
3.18.1. RAPD primers

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

<table>
<thead>
<tr>
<th>Seq Name</th>
<th>Primer Seq 5’-3’</th>
<th>Mer</th>
<th>TM</th>
<th>% GC</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAPD primers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPA1</td>
<td>CAGGCCCTTC</td>
<td>10</td>
<td>38.2</td>
<td>70%</td>
</tr>
<tr>
<td>OPA-4</td>
<td>AATCGGGCTG</td>
<td>10</td>
<td>39.3</td>
<td>60%</td>
</tr>
<tr>
<td>A-11</td>
<td>AGGGGTCTTG</td>
<td>10</td>
<td>31.8</td>
<td>76%</td>
</tr>
<tr>
<td>A-5</td>
<td>AGGGGTCTTG</td>
<td>10</td>
<td>36.8</td>
<td>73%</td>
</tr>
<tr>
<td>OPD6</td>
<td>GGGGTCTTGA</td>
<td>10</td>
<td>32.8</td>
<td>83%</td>
</tr>
<tr>
<td>AA-04</td>
<td>CAGGCCCTTC</td>
<td>10</td>
<td>38.2</td>
<td>70%</td>
</tr>
</tbody>
</table>

3.18.2. Amplification conditions

Temperature profile, 94°C for 4 min followed by 35 cycles 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.

3.18.3. Analysis of RAPD bands

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

3.18.4. 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.

3.18.5. 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.

3.18.6. 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.

3.19. 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.
3.19.1. ITS- PCR primers

The following primers were used to amplify ITS regions:

<table>
<thead>
<tr>
<th>Seq Name</th>
<th>Primer Seq 5’-3’</th>
<th>Mer</th>
<th>TM</th>
<th>GC %</th>
<th>Amplicon size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fusarium sp.</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fcg17F</td>
<td>TCGATATACCGTGCGATTTCC</td>
<td>21</td>
<td>65</td>
<td>47%</td>
<td>~570</td>
<td>Nicholson <em>et al.</em> (1998)</td>
</tr>
<tr>
<td>Fcg17R</td>
<td>TACAGACACCGTCAGGGGG</td>
<td>19</td>
<td>66</td>
<td>63%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Trichoderma sp.</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T/ITS1</td>
<td>TCTGTAGGTGAACCTGCGG</td>
<td>19</td>
<td>63.9</td>
<td>57%</td>
<td>~600</td>
<td>White <em>et al.</em> (1990)</td>
</tr>
<tr>
<td>T/ITS4</td>
<td>TCCTCCGCTTATTGATATGC</td>
<td>20</td>
<td>61.5</td>
<td>45%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.19.2. 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.

3.19.3. 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.

3.19.4. Sequence analysis

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

3.19.5. Chromatogram of sequence

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

3.19.6. Editing and alignment of sequence data

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

3.19.7. 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.

3.19.8. 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.

3.20. Extraction and Assay of defense enzyme activity

3.20.1. β-1, 3-glucanase (E.C. 3.2.3.39)

Extraction of β-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 β-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.

3.20.2. 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 ºC the
reaction was stopped by centrifugation at 10,000g for 3 minutes. 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 1 hour. 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 3 minutes 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 ºC.

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.

3.20.3. 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 ßmercaptoethanol 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 30 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.

3.20.4. 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 °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 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 H₂O₂ (Chakraborty et al., 1993). Specific activity was expressed as the increase in absorbance at 460 nm g⁻¹ tissue/ min⁻¹.

3.21. Transmission Electron Microscopy
3.21.1 Specimen preparation
3.21.1.1 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.

3.21.1.2 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).

3.21.1.3 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.

3.21.1.4 Embedding
The samples were dipped in LR White and kept overnight at 4° C. They were kept at room temperature for 3 hrs. A fresh change of LR white was done and kept at 56° C for 36 hrs.

3.21.2 Viewing preparation
3.21.2.1 Trimming
Moulds containing the samples were roughly trimmed with a block trimmer (Reichert TM 60) fitted with a rotating milling cutter.
3.21.2.2 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.

3.21.3 Immunogold labeling

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

3.21.3.1 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.

3.21.3.2 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.

3.21.3.3 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.

3.21.3.4 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.