Chapter 3
MATERIALS AND METHODS

3.1. Plant material

3.1.1. Collection of seeds

Rice seeds were collected from different regions of West Bengal and Sikkim. Brimful, Champasari and Black Nuniya from Bijanbari (27° 02'N 88° 07'E/ 27.04° N 88). Kaberi 9090, Loknath 505 and Gouraknath 509 from Indo-Japan hybrid seed centre, Siliguri (26.7100° N, 88.4300° E). Sano masuri and Adde from Sikkim (26.7100° N, 88.4300° E). Attheu and Maiti from Kalimpong (N 27° 12'457'' E 88° 14' .574'') .Swarnamasuri and Tulai panji from Malda(25.0000° N,88.1500° E) and finally UBKV-1,UBKV-4 and UBKV-5 from Uttar Bangha Krishi Vishwavidyalaya (UBKV), Coochbehar (26° 24'15" N, 89° 23'5"E) respectively for initial morphological screening and comparative analysis of brown spot disease(Table 3)

Table3. Rice cultivars and its GPS location.

Sl. No	Rice Cultivars	Cultivar type	Origin	GPS Location
1.	Brimful	Ethnic	Bijanbari	27° 02'N 88° 07'E/ 27.04° N 88
2.	Champasari	Ethnic	Bijanbari	27° 02'N 88° 07'E/ 27.04° N 88
3.	Black Nuniya	Local	Bijanbari	27° 02'N 88° 07'E/ 27.04° N 88
4.	Kaberi 9090	Commercial	Siliguri	26.7100° N,88.4300° E
5.	Loknath 505	Commercial	Siliguri	26.7100° N,88.4300° E
6.	Gouraknath 507	Commercial	Siliguri	26.7100° N,88.4300° E
7.	Sano Musuri	Ethnic	Sikkim	27.3300° N,88.6200° E
8.	Adde	Ethnic	Sikkim	27.3300° N,88.6200° E
9.	Attheu	Ethnic	Kalimpong	27° 04'N 88° 28'E/27.06,88.47°
10.	Maiti	Ethnic	Kalimpong	27° 04'N 88° 28'E/27.06,88.47°
11.	Swarnamasuri	Local	Malda	25.0000° N,88.1500° E
12.	Tulaipanji	Local	Malda	25.0000° N,88.1500° E
13.	UBKV-1	Research	UBKV	26° 24'15" N, 89° 23'5"E
14.	UBKV-4	Research	UBKV	26° 24'15" N, 89° 23'5"E
15.	UBKV-5	Research	UBKV	26° 24'15" N, 89° 23'5"E

3.1.2. Viability test

To check the viability of the seeds, they were initially surface sterilized with 0.1% (w/v) $HgCl_2$ for 3-4 minutes, and then washed with sterile distilled water and then

transferred to sterile Petri plates under aseptic conditions. The seeds were allowed to germinate for one week and percentage of germination was observed for each rice cultivars (Figure 2) .The seeds were stored in air tight bags at -4˚C for further use.

Figure 2: Rice cultivars grown in petriplates for viability test

3.1.3. Maintenance of rice seedlings in glass house

Fifteen different rice cultivars were given for germination and maintained in glass house of Immuno- phytopathology Laboratory, Department of Botany, University of North Bengal prior to its transfer to the experimental field. They were initially given for germination in earthen plates of 7 cm height and 16 cm diameter containing sandy

loam soil and farmyard manure in the proportion of 2:1 by weight. Rice nursery was used for transplanting within 25-30 days after the date of sowing (Figure 3).

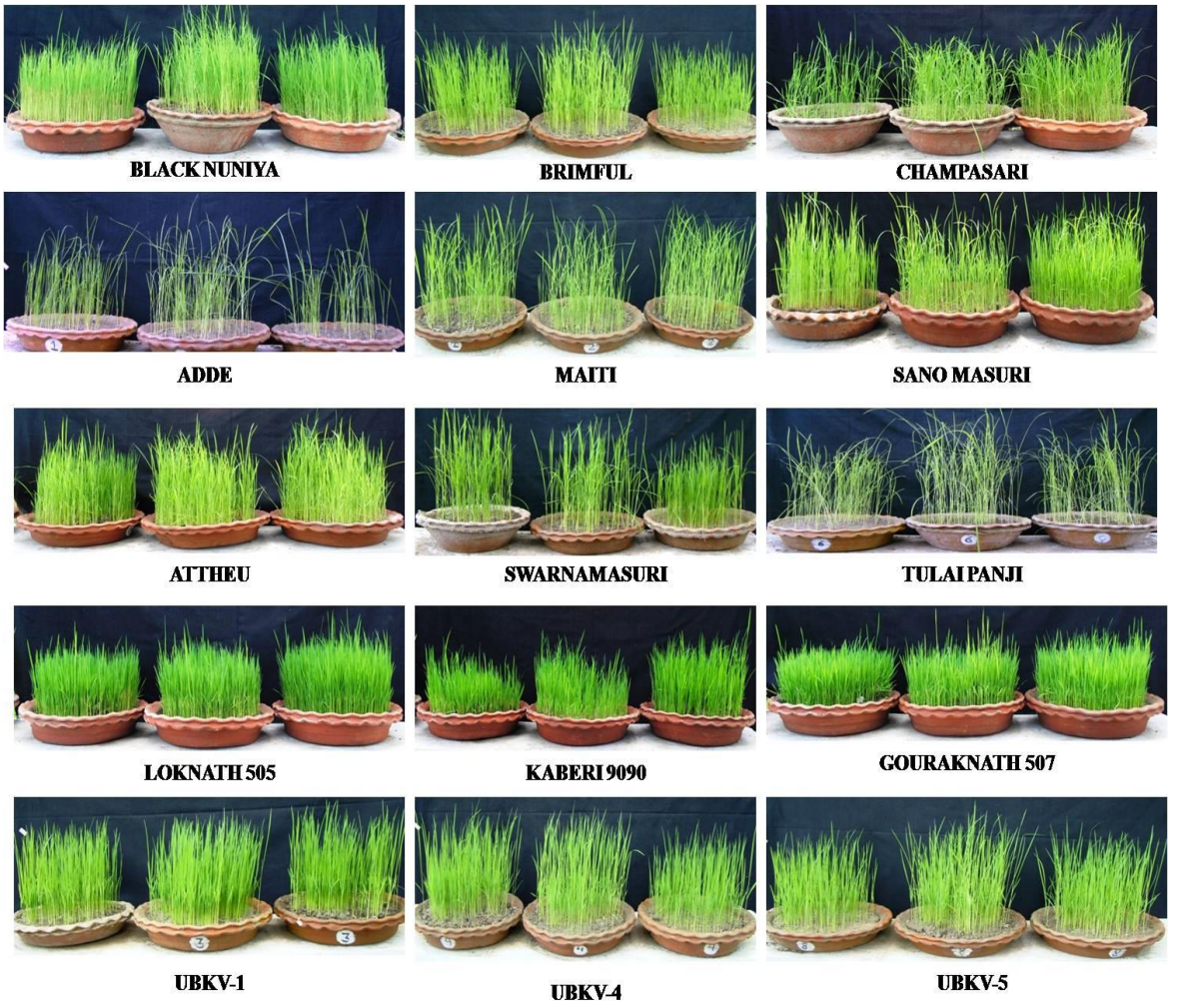

Fig 3: Maintenance of rice seedlings in glass house.

3.1.4. Maintenance in field condition

After germination the rice seedlings were then transferred to experimental fields of Immuno - Phytopathologyl Laboratory, Department of Botany, University of North Bengal. Eight inch row to row and plant to plant distance was maintained for each cultivar. Each block consisted of 10 lines of each cultivar with 10 transplanted rice plants. Healthy samples were maintained in glass house condition. The plants were grown during June to December. Plants were watered regularly twice in a day and weeding was done once a week (Figure 4).

Figure 4: Maintenance of rice cultivars in the field

3.2. Fungal culture

3.2.1. Isolation and maintenance

Fungal pathogen was isolated from samples of diseased leaves of rice (*Oryza sativa*) plants grown in experimental fields of Immuno- phytopathology Laboratory, Department of Botany, University of North Bengal by culturing pieces of internal tissues. Infected leaves 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 28ºC for one week. The fungal mycelium grown was transferred to PDA slants and kept for further identification.

3.2.2. Morphological and microscopic observation

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

3.2.3. Completion of Koch's postulate

Healthy leaves of rice (*Oryza sativa*) plants (1 month old) were further inoculated with this isolated organism and incubated for a period of 4 weeks for completion of Koch's postulate .Subsequently, after the development of the disease the infected leaves 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 28ºC. At the end of two weeks, the re isolated organism was examined, compared with the original stock culture and its identity was confirmed.

3.2.4. Assessment of mycelia growth

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

3.2.4.1. Solid media

To assess the growth of fungal culture in solid media, the fungus was first grown on Petri dishes, each containing 20ml of PDA followed by incubation for 7 days at 28ºC. 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. ***Oats meal agar (OA) :***

Oatsmeal-200g (blended in 600ml water,heated to 40-45^0 C), Agar 20.2g(melted in 400ml water).Both were mixed up,filtered and then autoclaved for 90 min and supplemented with 300µg ml /L Oxytetracycline.

3.2.4.2. Liquid media

To assess the mycelial growth in liquid media the fungus was first grown on petriplates, each containing 20ml of PDA and incubated for 5-8 days at 28ºC. The mycelial block (5mm) from the actively growing region of the fungus in the petriplate was cut with sterilized cork borer and transferred to Erlenmeyer flask (250ml) containing 50 ml of sterilized Potato dextrose broth (PDB), and incubated for 7-10 days with constant stirring at room temperature.

3.3. Soluble protein

3.3.1. Extraction and estimation of soluble proteins

3.3.1.1. Fungal Mycelia

Mycelial protein was prepared following the protocol as outlined by Chakraborty and Saha (1994). The fungal mycelia were grown in 250 ml Erlenmeyer flask each containing 50 ml of potato dextrose broth (PDB) and incubated for 10 days at 30+ 1ºC for extraction of antigen, mycelial mats was harvested washed with 0.2% Nacl and rewashed with sterile distilled water. Washed mycelia were crushed with sea sand using a chilled mortar and pestle and homogenized with cold 0.05m sodium phosphate buffer (PH-7.2) supplemented with 0.85% NaCl, 10mM sodium meta bisulphite, PVPP (Polyvinyl pyrollidine Phosphate) and 0.5mM magnesium chloride in ice bath. The homogenate mixture was kept for 2h or overnight at 4ºC and then centrifuged at 10.000rpm for 30 min, at 4ºC to eliminate cell debris. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring in ice bath and kept overnight at 4ºC. After this period, the mixture was centrifuged (10,000rpm) for 30 minute at 4º C, the precipitate was dissolved in the same buffer (pH 7.2). The preparation was dialysed for 72h through cellulose tubing (sigma chemical co., USA) against 1L of 0.005 M sodium phosphate buffer (pH 7.2) with six changes. The

dialysate was stored at -20ºC and used as antigen from the preparation of antiserum and other experiment.

3.3.1.2. Leaf

Soluble protein was extracted from rice 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 Na2 S2 O5, 0.5 mM MgCl2 and 2mM PVP was added during crushing and centrifuged at 4ºC for 20 min at 12000rpm. The supernatant was used as crude protein extract.

3.3.2. Estimation of 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).

3.4. SDS-PAGE analysis of soluble proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for the detailed analysis of protein profile following the method of Laemmli (1970).

For the preparation of gel the following stock solutions were prepared:

3.4.1. 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% bisacrylamide was prepared in water. The pH of the solution was kept below 7.0. The stock solution was then filtered through Whatman No. 1 filter paper, kept in brown bottle and stored at 4ºC and used within one month.

(B) Sodium Dodecyl Sulphate (SDS)

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

(C) Tris Buffer

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

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

(D) Ammonium Persulphate (APS)

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

(E) Tris –Glycine electrophoresis buffer

Tris running buffer consists of 25 mM Tris base, 250 mM 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 1 L of distilled water.

(F) SDS gel loading buffer

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

3.4.2. Preparation of gel

Mini slab gel (plate size 8 cm x10 cm) was prepared for the analysis of protein patterns by SDS-PAGE. For gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5 mm thick spacers were placed between the glass plates at three sides and sealed with high vacuum grease and clipped tightly to prevent any leakage of the gel solution during pouring. 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 2 hours. After polymerization of the resolving gel was complete, overlay was poured off and washed with water to remove any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and the comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30 - 45 minutes. After polymerization of the stacking gel the comb was removed and the wells were washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris - Glycine buffer was added sufficiently in both upper and lower reservoir. Any bubble trapped at the bottom of the gel, was removed carefully with a bent syringe.

Name of the compound	**10 % Resolving gel (ml)**	**5% Stacking gel (ml)**
Distilled water	2.85	2.10
30 % acrylamide	2.55	0.50
Tris*	1.95	0.38
10% SDS	0.075	0.030
10% APS	0.075	0.030
TEMED**	0.003	0.003
*For 1.5 M Tris pH 8.8 in resolving gel and for 1 M Tris pH 6.8 in stacking gel ** N,N,N',N'- Tetramethyl ethylene diamine		

3.4.3. Sample preparation

Sample (50 µl) was prepared by mixing the sample protein (35 µl) with 1X SDS gel loading buffer (15 µl) in cyclomixer. All the samples were floated in boiling water bath for 4 min to denature the protein sample. The samples were immediately loaded in a pre-determined order into the bottom of the wells with a microliter syringe. Along with the samples, protein markers consisting of a mixture of six proteins ranging from high to low molecular mass (Phosphorylase b- 97,4000; Bovine Serum Albumin- 68,000; Ovalbumin- 43,000; Carbolic Anhydrase- 29,000; Soyabean Trypsin inhibitor- 20,000; Lysozyme- 14,300) was treated as the other sample and loaded in a separate well.

3.4.4. Electrophoresis

Electrophoresis was performed at a constant 15 mA current for a period of three hours until the dye front reached the bottom of the gel.

3.4.5. 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 fixed in glacial acetic acid: methanol: water (10:20:70) for overnight. The staining solution was prepared by dissolving 250 mg of Coomassie brilliant blue (Sigma R 250) in 45 ml of methanol. After the stain was completely dissolved, 45 ml of water and 10 ml 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 hours 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 became clear.

3.5. Preparation of antigens

3.5.1. Fungal antigen

3.5.1.1. Pathogen

Mycelial protein was prepared following the method as outlined by (Chakarborty and Saha, 1994). Mycelia mats were harvested from 7-10 days old culture and washed with 0.2% NaCl then again rewashed with sterile distilled water. Washed mycelia were crushed with sea sand using a chilled mortar and pestle and homogenized with cold 0.05 M sodium phosphate buffer (pH 7.2) supplemented with 0.85% NaCl, 10 mM sodium metabisulphite and 0.5 mM $MgCl_2$ in ice bath. The homogenate mixture was kept for 2h or overnight at 4 ºC and then centrifuged at 10.000rpm for 30 min at 4ºC to eliminate cell debris. The supernatant was collected and stored in -20 ºC and used as antigen for the preparation of antiserum.

3.5.1.2. Leaf

Leaf protein was extracted from rice 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 Na2 S2 O5, 0.5 mM

MgCl2 and 2mM PVP was added during crushing and centrifuged at 4ºC for 20 min at 12000rpm. The supernatant was used as antigen.

3.6. Raising of polyclonal antibodies

3.6.1. Rabbits and their maintenance

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

3.6.2. Immunization

Before immunization, normal sera were collected from each rabbits. For developing antisera, intramuscular injections of 1ml antigen(protein extracted) mixed with 1ml of Freund's complete adjuvant (Genei) were given into each rabbit 7 days after pre- immunization bleeding and repeating the doses at 7 days intervals for consecutive week followed by Freund's incomplete adjuvant (Genei) at 7 days intervals upto 12-14 consecutive weeks as required. Method of (Alba and Devay, 1985 and Chakraborty and Saha, 1994) was followed for immunization.

3.6.3. Bleeding

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

distributed in 1 ml vials and stored at -20ºC as crude antisera. The serum was used for Dot blots analysis and Enzyme Linked Immunosorbent Assay (ELISA).

3.7. Purification of IgG

3.7.1. Precipitation

IgG was purified as described by Clausen (1988). Crude antiserum (2 ml) was first diluted with two volume of distilled water and an equal volume of 4 M ammonium sulphate. The pH was adjusted to 6.8 and mixture was stirred for 16 h at 20°C. The precipitate thus formed was collected by centrifugation at 12000 rpm at 22°C for 1 h. Then the precipitate was dissolved in 2 ml of 0.02 M sodium phosphate buffer, pH 8.0.

3.7.2. 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

3.7.3. Fraction collection

At the top of the column, 2 ml 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 buffer (pH 8.0). The buffer was applied in the flask on which rubber connection from its bottom was supplying the 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 mixed into buffer (1) thereby producing a continuous raise in molarity. Ultimately, 40 fractions each of 5 ml were collected and the optical density values were recorded at 280 nm using UV-Vis spectrophotometer.

3.8. Immunological assays

3.8.1. Plate trapped antigen coated (PTA) - ELISA

Plate trapped antigen coated (PTA)-ELISA was performed following the method as described by (Chakraborty *et al.* 1995) with modifications. Antigen were

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

3.8.2. 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.

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

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

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.

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

Nitrocellulose membrane (Millipore, 7cm x10cm, Lot No. H5SMO 5255, pore size 0.45µm, Millipore corporation, Bedford) was first cut carefully into the required size and fix between the template with filter paper at the bottom. 0.5M carbonate-bicarbonate buffer (pH 9.6), 4µl, was loaded in each well and allowed to dry for 30 min at room temperature. Antigen (5µl) was loaded on to NCM and allowed to dry for 30 min at room temperature. Template was removed and blocking of NCM was done with 19% non fat dry milk (casein hydrolysate, SRL) prepared in TBST for 30-60 minutes on a shaker. Respective polyclonal antibody (IgG 1:500) prepared against that antigen

was added directly in the blocking solution and further incubated at 4 ºC for overnight. The membrane was then washed gently in running tap water for three min, thrice followed by washing in TBST (pH 7.4), (Wakeham and White, 1996). The membrane was then incubated in alkaline phosphatase conjugated goat antirabbit IgG (diluted 1:10,000 in alkaline phosphatase) for 2h at 37ºC. The membrane was washed as before. 10 ml of NBT/BCIP substrate (Genei) was added next and color development was stopped by washing the NCM with distilled water and color development was categorized with the intensity of dots.

3.8.3. Western blot

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

The following buffers were used for Western blotting-

(i) All the stock solutions and buffers used in SDS-Gel preparation for Western blotting were as mentioned earlier in SDS-PAGE protein.

(ii) Tranfer buffer (Towbin buffer): (25mM Tris, 192mM glycine 20% reagent grade Methanol, pH 8.3).

Tris- 3.03g; Glycine- 14.4g; 200 ml Methanol (adjusted to 1lit. with dist. Water).

(iii) Phosphate buffer Saline, PBS, (0.15M, pH 7.2)

Stocks

A. Sodium dihydrogen phosphate- 23.40g in 1000ml dist. water

B. Disodium hydrogen phosphate- 21.294g in 1000ml dist. water

280 ml of stock A was mixed with 720 ml of stock B and the pH was adjusted to 7.2. Then 0.8% NaCl and 0.02% KCl was added to the solution.

(iv) Blocking solution: 5% non fat dried milk + 0.02% sodium azide in PBS with 0.02% Tween 20.

(v) Washing buffers:

(a) Washing buffer-1: PBS

(b) Washing buffer-2: (50mM Tris-HCl, 150 mM NaCl, pH 7.5). Tris- 6.07 g; NaCl- 8.78g; made up to 1lit. with distilled water.

(vi) Alkaline phosphatase buffer:(100mM NaCl, 5mM $MgCl_2$, Tris- HCl, pH 9.5). Tris- 12.14g; NaCl- 5.84g; $MgCl_2$- 1.015g; made up to 1 lit. with double distilled water.

(vii) Substrate

NBT: 5mg NBT in 100µl of 70% N,N-dimethyl formamide

BCIP: 5mg BCIP in 100µl of 70% N,N-dimethyl formamide

Substrate solution was prepared by adding 66µl NBT and 33µl BCIP in 10ml alkaline phosphatase buffer or, 1 tablet of NBT/BCIP (Sigma Chemical, USA) in 10ml of double distilled water.

(viii) Stop solution: (0.5M EDTA solution in PBS, pH 8.0) EDTA sodium salt-0.0372g in 200µl distilled water, added in 50ml of PBS.

Blot transfer process

Following the SDS-PAGE, the gel was transferred in prechilled transfer (Towbin) buffer for 1h. The nitrocellulose membrane (BIO-RAD, 0.45µm) and the filter paper (BIO-RAD, 2mm thickness) were cut to gel size, wearing gloves and soaked in Towbin buffer for 15 min. The transfer process was done in Trans- Blot SD Semi-Dry Transfer cell (BIO-RAD) through BIO-RAD power pack. The presoaked filter paper was placed on the platinum anode of the semi-dry cell. A pipette glass (or glass rod) was rolled over the surface of the filter paper to exclude all air bubbles. The pre wetted membrane was placed on top of the filter paper and air bubbles were rolled out. The equilibrated gel was carefully placed on the membrane and air bubbles were rolled out. Finally another presoaked filter paper was placed on the top of gel and air bubbles were removed. The cathode was carefully placed on the sandwich and pressed to engage the latches with the guide posts without disturbing the filter paper stack. The

blot unit was run for 45 min at a constant volt (15V). After the run the membrane was removed and dried on a clean piece of 3mm filter paper for 1h. and proceeded for immunological probing.

Immunoprobing

Following drying, blocking was done by 5% non fat dried milk in a heat sealable plastic bag and incubated for 90 min with gentle shaking on a platform shaker at room temperature. Subsequently, the membrane was incubated with antibody (IgG) solution (blocking solution: PBS [1:1, v/v + IgG, diluted as 1:100 or as per requirement]. The bag was sealed leaving space for few air bubbles and incubated at 4°C overnight. All the processes were done with gentle shaking. Next day the membrane was washed thrice in 250 ml PBS (washing buffer -1). Final washing was done in 200ml washing buffer-2 to remove azide and phosphate from the membrane before enzyme coupled reactions. The enzyme, alkaline phosphatase tagged with antirabbit goat IgG (Sigma Chemicals) diluted (1:10,000) in alkaline phosphatase buffer, was added and incubated for 1h. at room temperature. After enzyme reaction, membrane was washed four times in washing buffer-2. Then 10ml substrate was added and the reaction was monitored carefully. When bands were observed up to the desired intensity, the membrane was transferred to tray of 50ml stop solution.

3.8.4. Fluorescence antibody staining and microscopy

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

3.8.4.1. 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.

3.8.4.2. Cross section of rice leaves

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

3.8.4.3. Localization of chitinase and glucanase by immunofluorescence

Indirect fluorescence staining of cross-section of tea leaves was done using FITC labelled goat antirabbit IgG following the method of Chakraborty and Saha (1994). Initially, cross sections of healthy and infected tea roots were cut and immersed in phosphate buffer saline (PBS), pH 7.2. These sections were treated with normal serum or antiserum diluted (1:50) in PBS and incubated for 1h. at room temperature. After incubation, sections were washed thrice with PBS-Tween (pH 7.2) for 15 min and transferred to 40µl of diluted (1:40) goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC). The sections were incubated for 30 min 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. Photographs were taken by scope photo instrument directly attached to a computer. Fluorescence of the root section was observed using Leica Leitz biomed Microscope with fluorescence optics equipped with UV-filter set-I-3 and photograph was taken.

3.9. Isolation of genomic DNA

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

3.9.1. 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	**Genomic DNA Buffer**	**CTAB Buffer**
50 mM Tris, pH 8.0 100 mM EDTA 100mM NaCl 1% SDS	10 mM Tris, pH 8.0 0.1 mM EDTA	2% CTAB 1.5% PVP K 30 1.4 mM Nacl 20 mM EDTA 100mM Tris HCL pH 8.0 0.1% B-mercaptoethanol

3.9.2. 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 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.

3.9.3. 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^oC 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.

3.9.4. Measure DNA Concentration using Spectrophotometer

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.

3.9.5. 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. Preparing an agarose gel involves melting a specified amount (0.8%) of agarose in 1X TBE buffer, cooling the solution, and pouring it into the gel casting tray with ethidium bromide. Gels solidify in 15-20 minutes.

3.9.5.1. 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.

3.9.5.2. Run gel electrophoresis for DNA fraction

The electrical lead of the gel tank was attached firmly and applied electric supply at constant current 90 mA and voltage 75 volt (BioRAD Power Pac 3000) at least for 90 minutes. 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.

3.10. ITS PCR analysis

Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. PCR was programmed with an initial denaturing at 94^{o}C for 5 min. followed by 30 cycles of denaturation at 94^{o} C for 30 sec, annealing at 59^{o}C for 30 sec and extension at 70^{o}C for 2 min and the final extension at 72 ^{o}C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µ1) 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.

3.10.1. ITS PCR primers

The following primers were used to amplify ITS regions:

ITS-Primers pairs	**Primer Seq 5'-3'**	**Mer**	**TM**	**% GC**
T/ITS 1	TCTGTAGGTGAACCTGCGG	19	63.9	57%
T/ITS4	TCCTCCGCTTATTGATATGC	20	61.5	45%

3.10.2. Amplification conditions

Temperature profile, 94^{o}C for 4 min. followed by 30 cycles of denaturation at 94^{o}C for 30 sec, annealing at 60^{o}C for 30 sec and extension at 70^{o}C for 5 min and the final extension at 72^{o}C for 7 min in a Primus 96 advanced gradient Thermocycler.

3.10.3. Sequencing of rDNA gene

The rDNA was used for sequencing purpose. DNA sequencing was done bi-directionally using the ITS primer pairs by Sci Genome, Kerela.

3.10.4. Sequence analysis

DNA sequence information was analyzed using bioinformatic algorithms tools e.g. Bioedit, MEGA 4.

3.10.5. Chromatogram of sequence

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

3.10.6. Editing and alignment of sequence data

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

3.11. BLAST analysis of Sequence

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.

3.12. 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.

3.13. Multiple sequence alignment and phylogenetic analysis

The sequenced PCR product was aligned with ex-type strain sequences from NCBI Gene Bank and established fungal taxonomy for identification. Sequences were aligned following the Clustal W algorithm (Thompson *et al.,* 1994). Phylogenetic inference was performed by the Neighbor Joining (NJ) method (Saitou and Nei, 1987). Bootstrap tests with 500 replications were conducted to examine the reliability of the interior branches and the validity of the trees obtained (Tamura *et. al*., 2007).

3.14. Assessment of disease caused by fungal pathogens on rice plants

3.14.1. Detached leaf

A detached leaf inoculation technique as described by Dickens and Cook (1989) was followed for artificial inoculation of leaves. Fresh, young, fully expanded rice leaves detached from plants were placed in trays lined with moist blotting paper.

Wounds were made on adaxial surface of each leaf with 26 G½ needle and inoculated with 20µl droplets of spore suspension (1.2 X 10^6 conidia ml^{-1}) of the fungus (prepared from 14 days old culture in PDA). Spore suspension was placed (2-4 drops $leaf^{-1}$) on the adaxial surface of each leaf with a hypodermis syringe on the wounds. In control sets drops of sterile distilled water were placed on the wounded leaves. Each tray was covered with a glass lid and sealed with petroleum jelly in order to minimize drying of the drops during incubation.

Percent drops that resulted in lesion production were calculated after 48, 72 and 96 hours of incubation as described by Chakraborty and Saha (1994). Observations were made based on 6 inoculated leaves for each treatment in average of three separate trials.

3.14.2. Whole Plant

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

The disease severity on plant leaves was recorded using the method of Adlakha *et al*. (1984). Results were always computed as the mean of observations of 25 well-established and fully grown 6 months old rice plants in average of three separate experiments.

3.15. *In vivo* testing for antagonism to fungal pathogens

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

Ten previously isolated characterized sequenced PGPR strains were taken for the antagonistic study against the pathogen *D. oryzae*. The bacterial strains with NBAIM Acc. No. and NCBI(Gen Bank) Acc. No. are as follows *Bacillus pumilus* (NAIMCC-B01483) (JF836847), *Bacillus pumilus* (NAIMCC-B01487) (JQ765579), *Bacillus pumilus* (NAIMCC-B01488) (JQ765580), *Burkholderia symbiont* (NAIMCC-B01489) (JQ765578), *Bacillus aerophillus* (NAIMCC-B01490) (KC603894),

Paenibacillus polymyxa (NAIMCC-B01491) (KC703775), *Bacillus methylotrophicus* (NAIMCC-B01492) (JQ765577), *Bacillus altitudinis* (NAIMCC-B01484) (HQ849482), *Bacillus altitudinis* (NAIMCC-B01485) (JF899300), *Enterobacter cloacae*(NAIMCC-B01486) (KC703974) which are coded as (BRHS/C1), (BRHS/T382), (BRHS/T384), (BRHS/P92), (BRHS/ B104), (BRHS/R72), (BRHS/P91), (BRHS/P22), (BRHS/S73), (BRHS/R71) accordingly, was collected from Immuno- Phytopathology Laboratory, Department of Botany, University of North Bengal were evaluated against leaf pathogens- *Drechslera oryzae* in dual culture using PDA 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 28º±2ºC and inhibition zone towards the fungal colony in individual plate was quantified. Results were expressed as mean of percentage of inhibition of the growth of the pathogen in presence of the bacterial isolates. For each test three replicate plates were used. These bacteria were tested for antagonism to *Drechslera oryzae*, and were used for further evaluation.

3.15.2. Antifungal test of Plant growth promoting fungi (PGPF)

The efficacy of BCA (*Trichoderma* sp.) collected from Immuno- Phytopathology Laboratory, Department of Botany, and University of North Bengal was tested *in vitro* for inhibiting growth of the pathogen (*Drechslera oryzae*) in dual culture using PDA. Six previously isolated, characterized and sequenced *Trichoderma* isolates were taken into consideration for this study. Out of these six, three were *T. harzianum*, viz RHS/S559 (NAIMCC-F-03288, HQ334997), RHS/S560 (NAIMCC-F-03289, HQ334995) and RHS/M511 (NAIMCC-F-03290, GQ995194), and the other three were *T. asperellum*, viz RHS/M512 (NAIMCC-F-03291, HQ265418), RHS/M517 (NAIMCC-F-03292, HQ334994) and RHS/M561 (NAIMCC-F-03293, HQ334996). 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 28ºC and inhibition zone towards the fungus colony in individual plate was quantified. Results were expressed as mean % of inhibition in presence of the fungal isolate.

3.16. Mass multiplication of bioinoculants and pathogen and their application

3.16.1. Arbuscular Mycorrhizal Fungi (AMF)

3.16.1.1. Isolation of AMF spores

Arbuscular mycorrhizal fungal spores were screened from soil samples of fifteen rice varieties rhizosphere by the wet sieving and decanting method (Gerdeman and Nicholson, 1963). Soil samples (100gm each of the root zone) were collected, suspended in water (1lt.) in order to obtain a uniform suspension. Soil clusters are carefully dispersed in the water and is kept for 10 minutes to settle down the heavy particles. Aqueous suspension was passed through a set of sieves of different pore size (200, 170, 150, 80, 50 µm) arranged one below t other. The spores were picked by the help of bristles / brushes and transferred to grooved slides or vials and observed under dissecting microscope. Few spores were stained with Melzar's reagent and studied under sterio-microscope. Healthy spores are separated by fine brush and are stored in autoclaved glass vials either in sterile distilled water or Ringer's Solution (8.6gm NaCl, 0.3gm KCl, 0.33gm $CaCl_2$ in one litre of boiled distilled water) at 4ºC for further study and observation. It is evident from various studies that each plant has multiple AM fungi population.

3.16.1.2. Identification of AMF spores

Spore samples were separated according to their morphology size, colour, shape, wall thickness, wall layers, and other accessory structures like hyphal attachment etc. for the purpose of identification. The spores were identified with the help of standard keys (Walker, 1981; Schneck and Perez, 1987). Spores were critically examined with special reference to variation in vesicles (size, shape, wall thickness, wall layer, position and abundance), hyphal branching patterns, the diameter, structure (especially near entry points) and the staining intensity of hyphae.

3.16.1.3. Spore count

Rhizosphere soil (100gm) was taken and suspended in 250ml water. Wet sieving and decanting method was used for isolation of spores. Total number of spores was then counted and spore percentage of different genera was obtained.

3.16.1.4. Histopathological studies of rice roots

Fungal association of AM fungi within the root tissues was observed according to Philips and Hayman (1970).Young roots from rice 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 (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).

3.16.1.5. Percent Root colonization

Percent root colonization was estimated by using slide method by (Giovannetti and Mosse, 1980). All the infected and uninfected segments of root tissue and the percentage of infection was calculated as follows AMF infection (%) = [infected root segments / total fragments of root taken] X 100.

3.16.1.6. Mass multiplication of AMF

Maize (*Zea mays*), was used for the mass multiplication of AM fungi. Black plastic pots (12inch) were filled with sterilized soil to discard the presence of other fungal propagules. After the plantation adequate water was given. Spores collected from rice rhizosphere were collected 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). 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 required spores were present. Approximately > 175 spores / 100gms could be considered as potent inocula for application.

3.16.2. Plant Growth promoting fungi (PGPF)

3.16.2.1. Selection of PGPF

Three isolate of *Trichoderma harzianum* (NAIMCC-F-03288, 03289, 03290) and another three isolate of *Trichoderma asperellum* (NAIMCC-F-03291, 03292, 03293) were selected as plant growth promoting fungi based on their performance as potential growth promoters as well as their biocontrol efficiency in field grown crops (Sunar *et al*., 2014).

3.16.2.2. Mass multiplication and application

3.16.1.1. Wheat bran culture

Inoculum of *Trichoderma harzianum* and *Trichoderma asperellum* was 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. harzianum* was mixed to give a concentration of 105 cfu/g of soil as described by Chakraborty *et al.* (2003).

3.16.1.1. Foliar spray

The selected PGPF was grown in 100ml PDA medium for 7-10 days till sporulation occured. The spores were then scraped off from the surface of the media with the help of inoculating needle and the spores were collected in sterile distilled water. The spore suspension in sterile distilled water at a concentration of 3X 10^4 spores/ml after the addition of a few drops of Tween -20 was sprayed until run off on the foliar part of the three months old plants after pruning. The spraying was done forth nightly till the new shoots started appearing. The spraying was done for 4 times after every 3 days interval of time.

3.16.3. Plant growth promoting rhizobacteria (PGPR)

3.16.3.1. Selection of PGPR

Ten efficient bacterial isolates [*Bacillus pumilus* (NAIMCC-B01483) (JF836847), *Bacillus pumilus* (NAIMCC-B01487) (JQ765579), *Bacillus pumilus* (NAIMCC-B01488) (JQ765580), *Burkholderia symbiont* (NAIMCC-B01489) (JQ765578), *Bacillus aerophillus* (NAIMCC-B01490) (KC603894), *Paenibacillus polymyxa* (NAIMCC-B01491) (KC703775), *Bacillus methylotrophicus* (NAIMCC-B01492) (JQ765577), *Bacillus altitudinis* (NAIMCC-B01484) (HQ849482), *Bacillus altitudinis* (NAIMCC-B01485) (JF899300), *Enterobacter cloacae* (NAIMCC-01486) (KC703974)] were selected as Plant growth promoting rhizobacteria based on their performance as potential growth promoters as well as their biocontrol efficiency in field grown crops (Sunar *et al.,* 2013).

3.16.3.2. Mass multiplication and application of PGPR

3.16.3.2.1. Soil drench

The bacteria were grown in NB for 48 hrs at 28^{o}C and centrifused 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 $3X10^6$ cfu ml^{-1}. The bacterial suspension was applied to the pots during transplantation of seedlings . Applications were done @ 0f 100 ml per experimental field at regular interval of one month for three months subsequently.

3.16.3.2.2. Foliar spray

The bacterial pellet suspended in sterile distilled water at a concentration of 3x106cfu ml-1 after the addition of a few drops of Tween -20 was sprayed until run off on the foliar part of the three months old plants after pruning. The spraying was done for 4 times after every 3 days interval of time.

3.16.4. Application of different bioinoculants under field condition

For field inoculation, initially chopped maize roots colonized with dominant spores of *Rhizophagus fasciculatus* (AMF) were applied in the experimental field before the transfer of the seedlings. One month following the application of AMF, root colonization status was examined. Then after 15 days of treatment various mass multiplied *Trichoderma* sp (PGPF) in wheat bran was applied in the field. Two weeks after application of PGPF soil drench application or foliar spray application of various PGPR was done for 16 days at 3 days interval of time.In case of joint treatment with all three bioinoculants, they were added to the field sequentially but in case of dual treatments (AMF+PGPF, AMF+PGPR and PGPF+PGPR) application was done accordingly. Growth parameters were finally recorder after two months of application of last treatment.

3.16.5. Inoculum preparation of pathogen

The brown spot pathogen, *Drechslera oryzae* was grown in 100ml PDA medium for 7-10 days till sporulation occured. The spores were then scraped off from the surface of the media with the help of inoculating needle and the spores were collected in sterile distilled water. The spore suspension containing $3X\ 10^4$ spores/ml with 0.01% Tween 20 was sprayed on to the treated as well as untreated plants and covered with plastic bags for 48hrs.

3.17. *In vivo* assessment of plant growth promotion

3.17.1. Assessment of plant growth following application of bioinoculants

Plant growth promotion in terms of height was recorded after 20, 40, 60 and 80 days of application of bioinoculants and total biomass of root and shoot in field grown plants .

3.17.2. Assessment of disease sevierity

Establishment of naturally and artificially occurring brown spot disease was observed and disease severity was assessed in terms of lesion number per leaf and infection index calculated as described by Adlakha *et al.* (1984). Ten diseased leaves were randomly selected and incidence observed and finally Percentage Disease Index (PDI) was calculated.

For percent disease index (PDI) calculation, the following formula was used- [(class rating x class frequency)/(total no. of leaves x maximum rating scale)] x 100. Disease severity was recorded using a score chart consisting of nine different scales (0 = no incidence, 1= Less than 1%, 2 = 1-3%, 3 = 4-5%, 4 = 6-10%, 5=11-15%, 6 = 16-25%, 7 = 26-50%, 8 = 51-75% and 9 = 100%) of infection prepared on the basis of percent diseased area of the total leaf area (IRRI, 2002).

3.18. Extraction and asssay of defense enzymes activities after application of bioinoculants

3.18.1. 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). Rice leaves samples (1g) was crushed were crushed in liquid nitrogen and extracted using 5ml of chilled 0.05M sodium acetate buffer (pH 5.0) by grinding at 4^{o}C using mortar and pestle. The extract was then centrifuged at 10000 rpm for 15 min at 4^{o}C and the supernatant was used as crude enzyme extract.

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

3.18.2. 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 modification. Rice leaves samples (1g) were crushed in liquid nitrogen and extracted using 5ml of chilled 0.1 M Sodium Citrate buffer (pH 5). The homogenates were centrifuged at 12,000 rpm for 10 min and the supernatant was used as cenzyme source.

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-PO_4 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.

3.18.3. Phenylalanine Ammonia Lyase (PAL) (E.C.4.6.1.5)

Extraction of PAL (E.C.4.6.1.5) was done 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.

Phenylalanine ammonia lyase activity in the supernatant was determined by measuring the production of cinnamic- acid from L-phenylalanine spectrophotometrically. The reaction mixture contained 0.3ml of 300µM sodium borate (pH 8.8), 0.3ml of 3µM L-phenylalanine and 0.5ml of supernatant in a total volume of 3ml. Following incubation of 1 hour 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 tissue.

3.18.4. 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 β mercaptoethanol under ice cold conditions. The homogenate was centrifuged immediately at 15000 rpm for 20 minutes at 4ºC. After centrifugation the supernatant was collected and after recording its volume was immediately used for assay or stored at -20ºC (Chakraborty *et al.*1993).

For determination of peroxidase activity, 100µl of freshly prepared crude enzyme extract was added to the reaction mixture containing 1ml of 0.2M sodium phosphate buffer (pH 5.4), 100µl of 4Mm H_2O_2, 100µl O- dianisidine (5 mg ml-1 methanol) and 1.7 ml of distilled water. Peroxidase activity was assayed spectrophotometrically at 460 nm by monitoring the oxidation of O- dianisidine in the 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}.

3.18.5. Isozyme analysis of peroxidase

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

3.18.5.1. Preparation of the stock solution

Solution A: Acrylamide stock solution (Resolving gel)

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

Solution B: Acrylamide stock solution (stacking gel)

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

Solution C: Tris- HCl (Resolving gel)

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

Solution D: Tris- HCl (Stacking gel)

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

Solution E: Ammonium persulphate solution (APS)

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

Solution F: Riboflavin solution

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

Solution G: Electrode buffer

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

3.18.5.2. Preparation of gel

For the polyacrylamide gel electrophoresis of peroxidase isozymes mini slab gel was prepared. For slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any trace of grease and then dried. 1.5 mm thick spacers were placed between the glass plates on three sides and these were sealed with high vacuum grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. 7.5 % resolving gel was prepared by mixing solution A: C: E: 79 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.

3.18.5.3. Sample Preparation

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

3.18.5.4. Electrophoresis

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

3.18.5.5. 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 Garber (1971). The gel was incubated in the aqueous (80 ml) solution of Benzidine (2.08 g), Acetic acid (18 ml), 3 % H_2O_2 (100 ml) for 5 minutes. The reaction was stopped with 7 % Acetic acid. After the appearance of clear blue coloured bands, analysis of isozyme was done immediately.

3.18.6. Extraction and estimation of total sugar content

Extraction of total sugar was done following the method of Harborne (1973).With minor modifications under normal room temperature and light conditions. One gm of leaf tissue were weighed and crushed with 10ml of 95% ethanol. The alcoholic fraction was evaporated off on a boiling water bath. The aqueous fraction was centrifuged at 10,000 rpm for 15 min and the supernatant was collected and stored at -4^{o}C.

Estimation of total sugar content was determined following the method given by Plummer (1978). 1ml of test solution was reacted with 4ml of Anthrone's reagent (0.2% anthrone in conc. H_2SO_4) and mixed properly. The reaction mixture was incubated for 10mins in a boiling water bath at about 100^oC taking proper precaution followed by cooling it under running tap water. The absorbance was measured at 620nm in a colorimeter and quantified using a standard curve of D- glucose.

3.18.7. Extraction and estimation of total chlorophyll content

Extraction of chlorophyll from leaves was done according to the method of Harbone (1973). 1g of leaf sample was homogenized in 80% acetone and filtered through Whatman No. 1 filter paper in a dark chamber. Addition of 80 % acetone from the homogenized sample was done repeatedly. The filtrate was collected and the total volume was made up to 10 mL using 80% acetone.

Estimation of chlorophyll was done by measuring the OD of the filtrate at 663 nm and 645nm respectively in a UV-VIS spectrophotometer (UV-VIS spectrophotometer 118 systronics) against a blank of 80% acetone and calculated using the formula as given by Arnon (1949).

Total chlorophyll = $(20.2\ A_{645} + 8.02\ A_{663})$ mg g $^{-1}$fresh weight

Chlorophyll a = $(12.7\ A_{663} - A_{645})$ mg g $^{-1}$fresh weight

Chlorophyll b = $(22.9\ A_{645} - 4.68\ A_{663})$ mg g $^{-1}$fresh weight

3.18.8. Extraction and estimation of Phenol content

3.18.8.1. Extraction of phenol

Phenol was extracted from the fresh young leaves following the method of Mahadevan and Sridhar (1982). One g of leaf tissues 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.

3.18.8.2. Estimation of Total phenol

Total phenol content was estimated by Folin Ciocalteau's reagent, following the method of Bray and Thorpe (1954). To 1 ml of the alcoholic extract, 1 ml of 1 N Folin

Ciocalteau's reagent followed by 2 ml of 20 % sodium carbonate solution was added in a test tube. The test tube was shaken and heated on a boiling water bath for 1 minute. After cooling, the volume of the reaction mixture was raised to 25 ml. Absorbance of the blue colored solution was measured at 650 nm. Quantity of total phenol was estimated using caffeic acid as standard.

3.19. HPLC Analysis of phenols and phytoalexin

3.19.1 Sample preparation

3.19.1.1 Phytoalexin

Extraction procedure of rice phytoalexin Phytocassanes and was followed as described by (Umemura *et al*., 2003). Twenty grams of samples (about 25 leaves) were cut into pieces and then shaken with 200 ml of ethyl acetate and 200ml of 0.1 N Na_2CO_3 (pH 10.5) for 18h. The ethyl acetate fraction was collected and then mixed with 20ml of 0.02 N HCl and centrifuged at 15,000 × 30 min.

3.19.1.2. Phenolic compounds

Phenol extraction and preparation of the sample for HPLC was done by the method described by Pari and Latha (2004) in the dark. Fresh leaf tissue were taken and chopped into small pieces and soaked overnight in absolute methanol at solid material to methanol ratio of 1:3 (w/v) in dark. The suspension was filtered and the filtrate was evaporated using a rotary evaporator at 40^{o}C and lyophilized. It was re dissolved in 1mL of HPLC grade methanol and filtered through Millipore membrane (0.45μm) filter.

3.20. HPLC analysis

3.20.1. Phytoalexin

To measure the amount of phytoalexin especially Phytocassanes induced, the supernatant collected after the extraction procedure was put through High Performance Liquid Chromatography (HPLC) eluted with 45 % acetonitrile. (UV-VIS Detector and Liquid Chromatogram, SHIMADZU). Phytocassanes were monitored at 280 nm (Koga *et al*., 1998).

3.20.2. Phenolics

For the analysis of total phenol in HPLC a method followed by Pari *et al*., (2007) was used. For the HPLC finger print analysis of phenolic compounds present in extracts a Shimadzu system (Shimadzu Corp., Kyoto, Japan) was used, a flow rate of

1mL/ min, and gradient elution of HPLC grade of acetonitrile-water-acetic acid (5:93:2, v/v/v) [Solvent A] and of acetonitrile-water-acetic acid (40:58:2, v/v/v) [Solution B], a 0-50 min solvent B from 0-100%; and injection volume of 20μL were applied; whereas the separation of compounds was monitored at 280 nm. The identification and quantification of the phenolic compounds were done using the standards such as caffeic acid, ferulic acid, gallic acid, phloroglucinol, pyrogallol, resorcinol, salicylic acid and vannilic acid.

3.21. Bioassay of antifungal compounds

3.21.1. Radial growth bioassay

Radial growth inhibition bioassay was performed for determining antifungal activity of phytoalexin (Phytocassanes) extracted from rice leaves as described by Van Etten (1973).The sample (50µl) were taken in sterile petridishes (2" dia) and allowed to evaporate. Subsequently 10ml sterilized PDA medium was poured in each petridish, thoroughly mixed and allowed to solidify. Agar block (4 mm dia) containing mycelia of *Drechslera oryzae* (14 days old culture) were taken from the advancing zone and transferred to each Petri dish these were incubated at 28±2^{o}C, until inhibition of mycelia growth was observed. Percentage of mycelial inhibition was calculated using the following formula:

$$\text{Percentage of mycelia inhibition} = (C-T)/C \times 100$$

Where, C and T are the growth diameter (mm) in control and treated samples respectively.