CHAPTER 3
MATERIALS AND METHOD

3.1. Griding of study area

North Bengal has a total area of 21763.0 sq km stretching from $24^0$40’28’’ N to $27^0$13’ N Latitudes and $87^0$45’50’’ to $89^0$54’35’’ E Longitudes. The entire area comprises of six districts and three important ecological zones.

3.1.1. Darjeeling hill region

Darjeeling district is situated within the state of West Bengal. Kalimpong, Kurseong and Siliguri, are the sub-divisional headquarters of the district. The Hill areas of Darjeeling District are located within the lesser and Sub - Himalayan belts of the Eastern Himalayas. The area is bounded by the Sikkim Himalaya in the north, the Bhutan Himalaya in the east and Nepal Himalaya in the west. The southern foothill belt is demarcated by a highly dissipated platform of terrace deposits extending along the east west axis. The inner belt is defined by a ridgeline stretching from the Darjeeling Hill to the west and Kalimpong Hill to the east, overlooking the southerly flowing Tista valley in between. Prominent rivulets contributing to the Rammam - Rangit basin dissipate the northern slope of Darjeeling Hills. Geographically, the district can be divided into two broad divisions, the hills and the plains. The hilly region of the district are the three hill subdivisions of Darjeeling, Kurseong and Kalimpong. The foothills of Darjeeling Himalayas, which comes under the Siliguri subdivision, is known as the Terai. The major rivers flowing through here are- Teesta River , Mahananda River, the Great Rangit, Mechi, Balason, Lish, Gish, Chel, Ramman, Murti and Jaldhaka river. The Darjeeling hill area is formed of comparatively recent rock structure that has a direct bearing on landslides. Soils of Darjeeling hill areas are extremely varied, depending on elevation, degree of slope, vegetative cover and geolithology. As regards the geology of the land, Darjeeling Hill area represents a unique geo- environmental perception. It is primarily composed of erosional landforms produced by southerly flowing streams, which have exposed a full cross section of different tectonic units. The form units are, however

approximately the same throughout the hill area, having more or less uniform lithology, structure, climate, soil and vegetative covers. The contact between different groups of rocks is represented by thrusts, dipping at high angles towards north. There is various land formations found across the length of the Darjeeling Himalayas. These include Raised terraces, the Siwaliks, the Damuda series, Daling series and Darjeeling Gneiss. The soils of Darjeeling Hill area have developed depending upon the underlying geological structure. But, in general the soils have been developed by both fluvial action and lithological disintegration. The soils that have developed in the Kalimpong area are predominantly reddish in color. Occasional dark soils are found due to extensive existence of phyllitic and schists. Soils in the highlands stretching from the west to the east of the district along most of the interfluvial areas are mainly mixed sandy loam and loamy, while those on the southern slopes of Mirik and Kurseong are mainly clayey loam and reddish in color. Sandy soils are mainly found in the east of the river Tista. The basic soil types are yellow soils, red brown soils and brown forest soils. Red soil and yellow soil have developed on gneiss while brown on schists and shales. Coarse pale yellow to red brown soils are found on the Siwaliks while clayey dark soils are developed on Daling series. On the Darjeeling gneiss, very coarse-grained (50 percent -80 percent) particles are found. All the soils are definitely acidic in nature with the tendency to increase slightly in depth in most cases indicating the absence of bases. Gross cultivated in Darjeeling district is aroung 63, 786 ha. Important cash crops include Orange, Ginger and Cardamom. Apart from these Tea is on of the most important plantation crop of this region. There are 87 tea gardens spread across the Darjeeling hill which covers roughly 19,000 hectares of the total land area. The major portions of the forests are today found at elevations of 2000 meters and above. The area located in between 1000–2000 meters is cleared either for tea plantation or cultivation. The four major forest types according to altitudinal variation found in Darjeeling Hill Areas are: Tropical moist deciduous forest (300-1000mts);Tropical evergreen lower montane forest (1000-2000mts.) ;Tropical evergreen upper montane forest (2000-3000mts.) ;Temperate forest (3000-3500mts.) ;Sub temperate forest (above 3500mts.).About 30% of the forest covers found in the lower hills are deciduous. Evergreen forest constitutes only about 6% of the total forest coverage. *Shora robusta* remains the most prominent species of Tropical moist deciduous forest

along with heavy under growth. In the slopes on southern portion of the Tista and the Great Rangit valley and in the Goke forests, this type is found. These species cannot thrive in areas of lower precipitation. Tropical lower montane evergreen forests are found on steep higher slopes, where drainage condition is good; Dhupi (*Cryptomaria Japonica*) is a known variety. The impact of man on this variety is very conspicuous. Tropical upper montane evergreen forests are found in the areas where high humidity along with dense fogs and less sunlight is available. Undergrowth is dense and contains Nettles, Raspberries, Ferms and bamboos. On the steep ridges, Rhododendrons and bamboos are abundant.

3.2. Soil sampling strategy

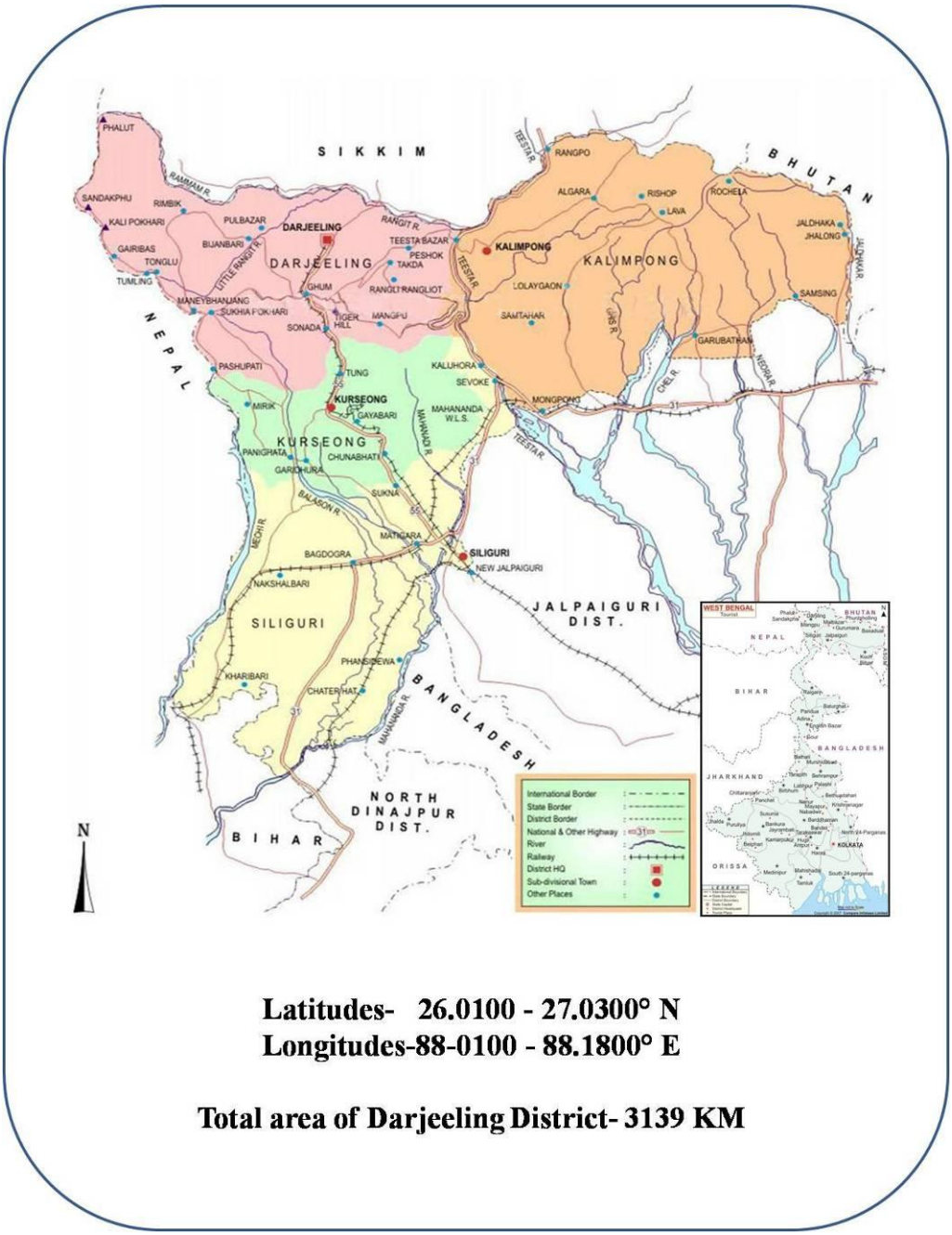
3.2.1. Sampling protocol

Each ecological zone (Terai and Dooars) of North Bengal have been divided into agriculturally dependent riverine and forest areas according to land use types. One of the most important steps in soil sampling is to collect the sample that represents that area, which means that the sample should be representative. Hence during sampling each zone has been divided into non-uniform random sampling units according to the type of vegetation they represent. While sampling the following points were taken into consideration:

(a) Use of proper sampling tools. (b) Avoiding unusual areas for sampling. (c) Dividing the areas for random sampling. (d) Taking composite sample from each area and (e) Taking proper records of the samples.

3.2.2. Grid sampling in non uniform ecological zones

Many sampling units were not uniform and varied both horizontally and vertically along the landscapes therefore the eco zones were broken into grids with shorter distances between the sampling points. This allows the development of precise sampling maps for further analysis. Since the sampling region falls under varying altitudes while determining the grid, one of the important things that have been considered was the altitude of the sampling unit areas.



Latitudes- 26.0100 - 27.0300° N
Longitudes-88-0100 - 88.1800° E

Total area of Darjeeling District- 3139 KM

Fig. 4. Map of Darjeeling district showing four main subdivisions

3.3. Isolation of microorganisms from soil

The following plating techniques were adopted for isolation of microorganisms from the collected soil samples

3.3.1. Soil dilution technique

Warcup's soil plate method (1955) for isolating microorganisms from the rhizosphere was followed with a few modifications. This is one of the most popular

methods for isolation and enumeration of soil borne actinomycetes, bacteria and fungi. It favors fungi that sporulates profusely or exist primarily as spores.

3.3.2. Direct soil plating

The process of Thomas and Parkinson (1965) has been adopted with modifications to isolate the fungi. Fungi that don't sporulate and exist as mycelium in soil seldom are isolated by the soil plating method. Soil (5-15 mg) was placed on a sterile culture plate and spread evenly; then 10-15 ml of molten agar medium was added. Finally soil particles were dispersed evenly with swirling motion.

3.3.3. Soil washing technique

Microorganisms not readily isolated from the soil plating technique were obtained from this technique of Watson (1960) where 1 g (air dried equivalent) of soil sample and 200 ml of sterile water were taken in a 500 ml flask, agitated with a blender and allowed to stand for 1-2 minutes, water was poured off and the process was repeated for 30-40 times further agitating by hand. After the final washing step the soil dilution plate method was followed.

3.4. Composition of Solid media

(A) For isolation of PGPR

Nutrient agar medium (NA)

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

King's B (KB)

Peptone 20.0 g,
K_2HPO_4 1.5g,
$MgSO_4.7H_20$ 1.5 g
Glycerol 15 ml
Distilled H_20- 1L,
pH 7.4 ± 0.2

(B) For isolation of *Trichoderma* species:

Special Nutrient Agar (SNA) (Samuels *et al.* 1998)

KH_2PO_4 1.0g
$MgSO_4.7H_2O$ 0.5g

KCl	0.2g
KNO_3	0.5g
Glucose	0.2g
Sucrose	1.0g
Agar	20.0g
Distilled water	1000ml

After sterilization, medium was supplemented with 300µg ml¯1L Oxytetracycline.

***Trichoderma* Selective Medium C (TSMC) (Elad *et al.*, 1981)**

$MgSO_4(7H_2O)$	0.2g
KH_2PO_4	0.9g
KCl	0.14g
NH_4NO_3	1.0g
Anhydrous Glucose	3.0g
Rose Bengal	0.15g
Agar	20.0g
Distilled water	950 ml

After autoclaving, 50 mL of anti microbial agents (Chloramphenicol 0.25g; Quintozone 0.2g; Captan 0.2g and Metalaxyl 1.6g) was added.

Cellulose Agar Medium (Kuling *et al.*, 2000)

Cellulose powder	30.0g
$NaNO_3$	3.0g
$(NH_4)SO_4$	1.0g
KH_2PO_4	1.0g
$(NH_4)_2HPO_4$	0.5g
$MgSO_4.7H_2O$	0.5g
$MnSO_4.6H_2O$	0.02g
Bacto yeast extract	0.3g
$FeSO_4.7H_2O$	0.1g
$CoCl_2.6H_2O$	0.02g
KCl	0.5g
Agar	18.0g

pH (before autoclaving) 6.5

After sterilization, supplemented with 300µg ml¯1L Oxytetracycline.

(C) For identification purpose:

Malt Extract Agar (MEA)

Malt extract	20.0g
Agar	20.0g

Water	1000ml

Oatmeal Agar (OA)

Oatmeal	200g (blended in 600ml water, heated to 40-45^{o} C)
Agar	20.0g (melted in 400ml water)

Both were mixed up, filtered and then autoclaved for 90 min and supplemented with 300µg ml^{-}1L Oxytetracycline.

Cornmeal Dextrose Agar (CMD)

Cornmeal	40.0g
Dextrose	20.0g
Water	1000ml

Filtered before autoclaving for 15min.

Difco cornmeal-dextrose agar 2% (w/v) supplemented with 300µg ml^{-}1L Oxytetracycline.

(D) For maintenance of cultures:

Potato Dextrose Agar (PDA)

Potato	200g
Dextrose	30.0g
Agar	20.0g
Water	1000ml
pH	6.5

After sterilization, PDA was supplemented with 300µg ml^{-}1L Oxytetracycline

(E) Richards agar (RA):

KNO_3	1.0g
KH_2PO_4	50g
$MgSO_4.\ 7H_2\ O$	0.25g
$FeCl_3$	0.002g
Sucrose	3.0g
Agar	2.0g
Distilled H_20	100ml

(F) Carrot juice agar (CJA):

Grated carrot	20.0g
Agar	2.0g
Distilled water	100 ml

(G) Czapek dox agar (CDA):

$NaNo_3$	0.20g
$KHPO_4$	0.10g
$MgSo_4.7H20$	0.05g
KCl	0.05g
$FeSo_4.7H20$	0.05g
Sucrose	3.0g
Agar	3.0g

Distilled water 100ml

(H) Potato sucrose agar (PSA):

Peeled potato 40.0g
Sucrose 2.0g
Agar 2.0g
Distilled water 100ml

(I) Malt extract peptone agar (MPA):

Malt extract 20.0g
Peptone 1.0g
Dextrose 20.0g
Agar 20.0g
Distilled water 1L

(J) Yeast extract dextrose agar (YDA):

Yeast extract 7.50g
Dextrose 20.0g
Agar 15.0g
Distilled water 1L

(K) Flentze's soil extract agar (FSEA):

Soil extract 1L
Sucrose 1.0g
KH_2PO_4 0.20g
Dried yeast 0.10g
Agar 25.0g

3.5. Morphological and Microscopical Characterization of isolates

3.5.1. Assessment of mycelial growth.

Mycelial growth of the fungal cultures was assessed in both on solid media and liquid media to know their culture characteristic.

3.5.1.1. 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.

3.5.1.2. 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 30 ºC. The mycelial block (5mm) from the actively growing region of the fungus in the petriplate was cut with sterilized cork borer and transferred to Ehrlenmeyer flask (250ml) containing 50 ml of sterilized liquid media Potato dextrose broth (PDB), Richards medium and Nutrient broth (NB) and incubated for 6 - 8 days with constant stirring at room temperature. After incubation the mycelia were harvested through muslin cloth, collected in aluminium foil cup of known weight and dried at 60ºC for 96 hour, cooled in desiccators and weighed.

3.5.2. Assessment of bacterial growth

For assessment of bacterial growth in liquid medium for 1ml of bacterial suspension was inoculated into the Nutrient broth medium (Peptone - 5.0g, Beef extract - 3.0g, NaCl - 5.0g, Yeast extract - 3.6g, Water - 1000ml, pH – 7.4 ± 0.2) and allowed to grow for 48h. following growth, absorbance was noted in a colorimeter at 600nm. Absorbance was converted into cfu/ml from a standard where known concentration of bacterial suspension was used. The cfu values were counted to log whenever needed.

3.5.3. Microscopical characterization

3.5.3.1. Bright field study of fungal spores and mycelia

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

3.5.3.2. SEM studies of fungal isolates

Selected microorganisms were examined under scanning electron microscopy (SEM). Samples were prepared according to a modification of the method described by King and Brown (1983). Test isolates were grown on PDA plates for l0 days in daylight at room temperature. Small pieces of the agar (less than 1 cm), with aerial sporulating culture attached, were excised from each plate and transferred to the

interior surface of a dry glass Petri dish lid. Efforts were made not to disturb the attached culture. Steps that involved exposing the samples to the atmosphere were performed quickly to minimise air-drying artifacts. The specimen dishes were then placed in vapour diffusion dehydration (VDD) assembly, and a vacuum was drawn. All samples were left in the VDD assembly where a maximum level of dehydration was achieved. The vacuum was released slowly and the specimen dish was removed from the desiccator. Each sample was placed within a separate aluminium “disc cup" (20 mm diam x 5 mm deep). Each sample was lifted from the bottom of the specimen dish with fine forceps and was positioned upright in a disc cup. The samples were then dried. All dried samples were mounted on double-sided tape affixed to SEM specimen mounts and were subsequently sputter-coated with gold. Gold coated samples were examined with a Philips 505 scanning electron microscope operating at 9.5-r5 Kev.

3.5.3.3. SEM studies of bacterial isolates

For scanning electron microscopy of the bacterial cells, 2 days old culture grown in nutrient broth medium were centrifuged at 3000 rpm. The pellet were collected and washed with 0.1 M phosphate buffer saline then the samples were prefixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer pH 6.8 under vacuum followed by dehydrolysis of the sample by different ethanol volumes starting; 30%, 50%, 70%, 80%, 90% and 100% and for each ethanol volume incubated for 10 minutes. After stepwise dehydration in graded alcohol, the samples were critical point dried in CO_2 (CPD 030; BAL TEC, Vaduz, Liechtenstein), mounted onto the sample stubs and were coated with 20 nm silver-palladium alloy in a mini sputter coater (SC7620) and examined in a JEOL JSM 5200 Scanning Electron Microscope (Tokyo Japan).

3.6. Biochemical characterization of bacterial isolates

3.6.1. Gram reaction

Gram reaction was carried out as outlined by Buchanan and Gibbson (1974). 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, and heat fixed. The smear was then flooded 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 becomes decolorised, 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.

3.6.2. Catalase

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

3.6.3. Urea digestion

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.

3.6.4. H_2S production

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

3.7. *In vitro* characterization of plant growth promoting activities

3.7.1 Phosphate solubilizing activity

3.7.1.1. Screening for primary phosphate solubilizing activity on PKV medium

Preliminary screening for phosphate solubilization was done by a plate assay method using Pikovskaya (PVK) agar medium (Pikovskaya 1948) 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.

3.7.1.2. Quantitative measurement of phosphate solubilization

Evaluation of phosphate solublizing activity of both the fungal and bacterial isolates were done by growing the isolates in the two sets of Pikovakaya's liquid medium

amended with 0.5% tricalcium phosphate and 0.5 % rock phosphate separately over a period of 10 days at 28^0C with constant shaking at 100 rpm in a rotary incubator. Quantitative estimation of phosphate is done following amonium molybdate ascorbic acid method as described by Kundsen and Beegle (1988). Amount of phosphate utilized or solubilized by the isolates were expressed as mg/L phosphate utilized by deducting the amount of residual total phosphate from the initial amount of phosphate source added to the modified Pikovakaya'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 with a pH meter fitted with electrode. The mycelia were harvested after 10 days of incubation by filtering and the change in the pH of the culture filtrate was recorded after centrifuging the medium at 5000 x g for 5 min. on a table centrifuge.

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

3.7.2. IAA production

3.7.2.1. Qualitative test for IAA production

10 ml of Davis Mingoli's broth supplemented with 0.1% tryptophan was inoculated with the isolate and incubated anaerobically at 37º C for 7 days. The culture was centrifuged at 10000 rpm for 15 min and supernatant was taken for analysis.

The supernatant was layered carefully with 2 ml of Ehrlich- Bobme (P-dimethylaminobenzaldehyde 10g, concentrated HCL 100ml) reagent on the surface, allowed to stand for a few minutes and observed for the formation of a ring at the supernatant- reagent interface indicating the production of indole.

3.7.2.2. Quantitative test for 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 $FeCl_3$ per litre in 7.9 M H_2SO_4 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. The amount of IAA produced was calculated with the help of a standard curve.

3.7.3. 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 $FeCl_3.6H_2O$ 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.

3.7.4. HCN production

Production of hydrocyanic acid was determined using the procedure described by Reddy *et al.* (2008) with slight modification. The selected bacterial isolates were grown at room temperature (37ºC) 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 in side the conical flasks in a hanging position. After incubation at 37ºC 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.

3.7.5. Chitinase production

3.7.5.1. Detection in modified Chitinase detection agar

For detecting the chitinolytic behavior of the bacteria chitinase detection agar (CDA) plates were prepared by mixing 1.0% (w/v) colloidal chitin with 15 g of agar in a medium consisted of (Na_2HPO_4 6.0 g, KH_2PO_4 3.0 g, NaCl 0.5 g, NH_4Cl 1.0 g, yeast extract 0.05g and distilled water 1 L; pH 6.5).

The CDA plate was spot inoculated with organism followed by incubation at 30°C for 7-10 days. Colonies showing zones of clearance against the creamy background were regarded as chitinase producing strains (Kamil *et al*. 2007).

The colloidal chitin was prepared by following the method described by Mathivanan *et al.* (1997), 5 g of chitin powder was slowly added to 60 ml of concentrated HCl and left at 4°C overnight with vigorous stirring. The mixture was added to 2 L of ice cold 95 % ethanol with rapid stirring and kept overnight at 25°C. The precipitation formed was collected by centrifugation at 7000 rpm for 20 min at 4°C and washed with sterile distilled water until the colloidal solution became neutral (pH 7). The prepared colloidal chitin solution (5 %) was stored at 4°C until further use.

3.7.5.2. Quantification of Chitinase activity

Spore suspension (1.0×10^6 spores per mL of culture medium of biocontrol agent were grown in 150 mL flasks containing 20 mL of unbuffered mineral synthetic medium (MSM) supplemented with dried mycelium as the sole carbon source (5 g L^{-1}). The cultures were grown at 30°C for 5 days without shaking. Culture filtrates were centrifuged at 4°C for 10 min at 5000 x g and the clear supernatants were either immediately tested for enzyme activity or stored at –20 °C until assayed.

Chitinase activity was assayed using the colorimetric method described by Molano *et al.* (1977) with minor modifications (Ulhoa, 1992*)*. The assay mixture contained 1 mL of 0.5 % pure chitin (suspended in 50 mM acetate buffer pH=5.2) and 1 mL of enzyme solution. The reaction mixture was incubated for 12 h at 37 °C with shaking and was stopped by centrifugation (5000 g/min) for 10 min and the addition of 1 mL of dinitrosalicylate (DNS) reagent (Miller, 1959).

3.7.6. Protease production

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

3.7.7. Starch hydrolysis

Detection of starch hydrolysis was detected by streaking the isolate on sterilized starch agar plate (NA + 0.1% soluble starch) and incubating for five days at 37º C. The plates were flooded with Lugol's iodine solution. The clear zone underneath and around the growth indicates the starch hydrolysis.

3.8. Screening for cellulase production

Microorganism showing cellulase activities were screened in the medium containing only cellulose as the carbon source. Both exo and endo cellulase activities were determined as the amount of glucose released from the substrate. The amount of glucose released by exocellulase activity of the microorganism during the growth period was measured following the DNS method of Miller (1972). To determine endo and exo β-1, 4 glucanase activity and the amount of glucose released in the medium corresponding to the amount of substrate utilized combined assay was conducted using filter paper assay (FPA) method of Miller (1972).

3.8.1. Assay of endocellulase activity

The amount of glucose released by endocellulase activity of fungal hyphae during their growth using cellulose as C source was measured following the DNS method of Miller (1972) where the culture filtrate was collected from the fermentation media by centrifugation. Culture filtrate (1 ml) was taken in a test tube and equalized with 2ml of distilled water. To the prepared culture filtrate, 3 ml of DNS reagent was added. The contents in the test tubes are heated in a boiling water bath for 5 min. After heating, the contents were allowed to cool at room temperature. At the time of cooling, 1 ml of freshly prepared 40% sodium potassium tartarate solution was added. After cooling, the absorbances were recorded at 510 nm in a U.V. vis spectrophotometer. The amount of reducing sugar was determined using a standard graph.

3.8.2. Assay of both exo and endocellulase activity

A combined assay for endo and exo cellulose activity in culture filtrate is carried out by FPA (Filter Paper Assay). The substrate used is Whatman No. 1 filter paper which was homogenized in 0.2 M sodium acetate buffer, pH 5.5 (5 mg in 20 ml buffer). 0.5

ml of culture filtrate was added to 2ml of substrate. The mixture was incubated at 35°C for one hour, 2 ml of DNS reagent was added and reaction was terminated. Then it was heated in a boiling water bath for 5 min following which 1 ml of potassium sodium tartarate (40%) was added to the warm tubes. The tubes were allowed to cool and the absorbance was recorded at 540 nm in a U.V. vis spectrophotometer.

3.9. Casein hydrolysis

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

3.10. *In vitro* testing for antagonism to fungal pathogens

3.10.1. Inhibition of mycelial growth in solid medium

For *in vitro* evaluation of antagonistic activity of both PGPR and fungal isolates, the following fungal pathogens viz., *Sclerotium rolfsii*, *Thanatophorous cucumeris*, *Rhizoctonia solani* and *Macrophomina phaseolina*. were used. The fungal pathogens were obtained from Immuno-Phytopathology Laboratory, Department of Botany, N.B.U. and were maintained with regular sub culturing in PDA for subsequent tests. Isolated microorganisms were tested for their *in vitro* antifungal activity against plant pathogens by dual inoculation technique. In case of fungal isolates, both the test organisms and the pathogens were grown separately in the petriplates and inocula were cut from the growing region and placed in fresh sterile PDA plates. In each plate, inoculum block of the isolate and of the test pathogen were placed 4 cm apart on the agar medium. The culture plates were seeded with the potential antagonist and the test pathogen at a distance determined by their growth rate (Klingstrom and Johansson, 1973). Interactions were observed at different intervals from 4th day onwards. For each test three replicates were used.

Whereas in case of PGPR isolates, the bacteria were streaked on one side of the Petri plate containing Potato Dextrose Agar (PDA) medium and 5mm fungal pathogen block was placed at the other side of the plate at a distance of 5 cm, incubated for 2-7 days at 28º±2ºC and inhibition zone towards the fungal colony in individual plate was measured. Results were expressed as mean of percentage of inhibition of the growth of the pathogen in presence of the bacterial isolate. For each test three replicate plates were used.

3.10.2. Sclerotia germination bioassay

For assessing the effect of active principle from cell free culture filtrate of BCA and PGPR on sclerotial germination of *Sclerotium rolfsii*, the mature sclerotia were scrapped off from the culture grown on PDA medium. Sclerotia were then soaked in cell free culture filtrate for 1h and placed on sterile black filter paper which were also aseptically soaked in the culture filtrate for at least 30 min. The black filter paper was then placed in a sterile Petri plates. Sclerotia soaked in distilled water and uninoculated sterile PDB served as control. On each soaked filter paper 50 sclerotia were placed with at least three replicates. These were allowed to germinate for 2-4 days after which percent germination was determined.

3.11. Immunological studies

3.11.1. Preparation of fungal and bacterial antigen

Fungalmycelial and Bacterial cell protein were prepared following the method as outlined by Chakarborty and Saha (1994). In case of fungal isolates, mycelial mats were harvested from 5-7 days old culture, washed with 0.2% NaCl and 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 4ºC and then centrifuged at 10.000rpm for 30 min at 4ºC to eliminate cell debris. The supernatant was collected and stored in -20ºC and used as antigen for the preparation of antiserum.

Prior to injection quantity Total Soluble Protein was measured and these was also analyzed by 12 % SDS-PAGE.

3.11.2. 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% Na2 CO3 in 0.1 NaOH) was added. This was incubated for 15 minutes at room temperature and then 0.5ml of 1N Folin Ciocalteau reagent was added and again incubated for further 15 minutes following which optical density was measured at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

3.11.3. SDS-PAGE analysis of soluble proteins

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

3.11.3.1. Preparation of stock solution

Following stock solutions were prepared.

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

A stock solution containing 29% acrylamide and 1% bis-acrylamide was prepared in warm water, as both of them are slowly dominated to acrylic and bis acrylic acid by alkali and light. The pH of the solution was kept below 7.0 and the stock solution was then filtered through Whatman No. 1 filter paper and kept in brown bottle, stored at 4ºC and used within one month.

B. Sodium Dodecyl Sulphate (SDS)

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

C. Tris Buffer

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

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

D. Ammonium Persulphate (APS)

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

E. Tris- Glycine electrophoresis buffer

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

F. SDS gel loading buffer

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

3.11.3.2. Preparation of gel

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

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

Name of the compound	**Resolving gel**		**5% Stacking gel (ml)**
	10% (ml)	**12%(ml)**	
Distilled water	2.95	2.45	2.10
30% acrylamide	2.50	3.00	0.10
Tris*	1.90	1.90	0.38
10%SDS	0.075	0.070	0.030
10%APS	0.075	0.070	0.030
TEMED**	0.003	0.003	0.003

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

** N, N, N', N' –Tetramethyl ethylene diamine.

3.11.3.3. 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 3 minutes to denature the proteins. After boiling, the sample was loaded in a predetermined order into the bottom of the well with T-100 micropipette syringe. Along with the protein samples, a marker protein consisting of a mixture of six proteins ranging from high to low molecular mass (Phosphorylase b-97, 4000: Biovine Serum Albumin-68,000; Albumin-43,000; Carbolic Anhydrase-29.000; Soybean Trypsin inhibitor-20,000; Lysozyme-14,300) was similarly treated as the other samples and loaded in a separate well.

3.11.3.4. 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.

3.11.3.5. 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 hours at 37ºC 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 37ºC with constant shaking until the background become clear.

3.11.4. Raising of polyclonal antibodies

3.11.4.1. Rabbits and their maintenance

Polyclonal antibodies were prepared against fungal antigens in New Zealand white male rabbits 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.

3.11.4.2. Immunization

Before immunization, normal sera were collected from each rabbits. For developing antisera, intramuscularly 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. Methods of Alba and Devay (1985) and Chakraborty and Saha (1994) were followed for immunization.

3.11.4.3. 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 board fixed at an angle of 60º, and the rabbits were held 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 immunobinding assay and indirect immunofluorescence study.

3.11.5. Purification of IgG

3.11.5.1. 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 ammonium sulphate was taken and adjusted to pH to 6.8, the mixture was stirred for 16h at 20º C in magnetic stirrer. The precipitate thus formed was collected by Centrifugation at 12,000 rpm for 1h at 22ºC. Supernatant was discarded and pellet was used for further steps.

3.11.5.2. 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.

3.11.5.3. 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).

3.11.6. Immunodiffusion test

3.11.6.1. 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.

3.11.6.2. Diffusion

Agar gel double diffusion tests were carried out using antigen and antiserum following the meyhod of Ouchterlony (1976). Antigen plus undiluted Antisera appropriately diluted were poured into wells with sterile 60-8- micropipette (50µl/well^{-1}) antisera in middle. Slides were kept in moist chamber at 25C for 72h. Precipitations reaction was observed in the agar gel only in cases where common antigen was present.

3.11.6.3. Washing, staining and drying of slides

After immunodiffusion, the slides were initially washed with sterile distilled water and then with aqueous NaCl solution (0.9% NaCl and 1% NaN_3) for 72 h with 6 hourly changes to remove unreacted antigens and antisera widely dispersed in the agarose gel. Then the slides were stained with Coomassie brilliant blue (R250, Sigma: 0.25g Coomassie blue, 45ml methanol, 45ml distilled water and 10ml glacial acetic acid) for 10 min at room temperature. After staining, the slides were washed in destaining solution (methanol: distilled water: acetic acid in 45:45:10 ratios) with changes until background become clear. Finally slides were washed with distilled water and dried in hot air oven for 3 h at 50 º C

3.11.7. Immunoblotting

3.11.7.1. Dot immunobinding assay (DIBA)

Dot immunobinding assay 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 0h 7.4) with 0.9% NaCl and 0.5% Tween 20 for washing.
c. Blocking solutions 10% (w/v) skim milk powder (casein hydrolysate, SLR) in TBST (0.05 M Tris-HCl, 0.5 M NaCl) 5% v/v Tween 20 , pH 10.3.
d. Alkaline phosphatase buffer (100 mM tris HCl, 100 mM NaCl, 5mM $MgCl_2$

Nitrocellulose membrane (Millipore, 7cm x10cm, Lot No. H5SMO 5255, pore size 0.45µm, Millipore corporation, Bedford) was first cut carefully into the required size and fix between the template with filter paper at the bottom. 0.5M carbonate-bicarbonate buffer (pH 9.6), 4µl, was loaded in each well and allowed to dry for 30 min at room temperature. Load 5µl (antigen) test sample on to NCM and allow it to

dry for 30 minute 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 minutes, thrice followed by washing in TBST (pH 7.4), (Wakemen 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.

3.11.7.2. Western Blotting

Western Blotting has been performed following the method of Wakeham and White (1996). The following buffers were used for Western blotting.

Stock solutions

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

(ii) Transfer buffer (Towbin buffer) :
(25mM Tris, 192mM glycine 20% reagent grade Methanol, pH 8.3).
Tris-3.03g, Glycine -14.4g, 200 ml Methonal (adjusted to 1L, with dist. Water).

(iii) Phosphate buffer Saline, PBS, (0.15M, pH 7.2)
Preparation was as mentioned in ELISA.

(iv) Blocking solution
Casein hydrolysate -5% in PBS, Sodium azide -0.02%, Tween-20 - 0.02%.

(v) Washing buffers : Washing buffer -1 : PBS: Washing buffer -2 : (50mM Tris-HCl, 150mM NaCl, pH 7.5). Tris-6.07 gm; NaCl – 8.78gm; made upto 1 lit with distilled water.

(vi) Alkaline phosphatase buffer : (100mM NaCl, 5mM $MgCl_2$, Tris-HCl, pH9.5) Tris-12.14gm; NaCl, 5.84gm; $MgCl_2$ -1.015gm; made upto 1lit with double distilled water.

(vii) Substrate: NBT: 5mg NBT in 1ooµl of 70% N,N-dimethyl formamide BCIP: 2.5mg BCIP in 50µl of 100% N, N-dimethyl formamide. Substrate solution was prepared by adding 66µl NBT and 33µl BCIP in 10ml alkaline phosphatase buffer.

(viii) Enzyme. (Alkaline phosphatage tagged with antirabbit goat IgG) Alkaline phosphatase buffer; enzyme (1;10,000).

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

3.11.7.2.1. Extraction and estimation of protein:

Protein extraction and estimation was done as described earlier.

3.11.7.2.2. SDS PAGE of protein

SDS-PAGE was carried out as mentioned earlier.

3.11.7.2.3. Blot transfer process

After the gel run, the SDS-PAGE gel was transferred to pre-chilled (Towbin) buffer and equilibrated for 1h. The nitrocellulose membrane (BIO-RAD, 0.45 µm) and the filter paper (BIO-RAD, 2mm thickness) were cut to gel size, wearing gloves, and soaked in Towbin buffer for 15min. 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. And 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 with a glass rod. 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. 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 1 h. and proceeded for immunological probing.

3.11.7.2.4. Immunoprobing

After drying the NCM for at least 1h, blocking of the unbound sites of NCM 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 require ment]. 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 withantirabbit 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 for 3 times in washing buffer-2.Then 10ml substrate was added and the reaction was monitored carefully. When bands were observed upto the desired intensity, the membrane was transferred to tray of 50ml stop solution.

3.11.8. Fluorescence antibody staining and microscopy

Indirect fluorescence staining of fungal mycelia was done using FITC labeled goat antirabbit IgG following the method of Chakraborty and Saha (1994). Fungal mycelia were grown in liquid Richards's medium as described earlier. After four days of inoculation young mycelia were taken out from flask and taken in Eppendorf tube and was 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 hour at RT. The mycelia washed thrice with PBS- Tween pH 7.2 as mentioned above and treated with Goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC-conjugate) (Sigma chemicals) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 45 minute 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.

3.11.9. Immunolocalization of Chitinase enzymes by Indirect immunofluorescence staining of leaf, stem and root tissues

Localization of Chitinase enzyme expression in the leaf, stem and root tissues of different test crops were conducted following the method of Chakraborty and Saha, 1994. Cross section of healthy, infected and treated roots were cut and immersed in PBS, ph7.2. These section were treated with primary antibody raised against Chitnasse enzyme which was diluted (1:50) in PBS and incubated for 1 hour at RT. After incubation, section were washed thrice with PBS- Tween pH7.2 for 15 minute

and transferred to 40µl of diluted (1:40) goat antirabbit IgG conjugated with fluorescence (FITC).The sections were incubated for 30 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 tissue sections were observed using Leica Leitz Biomed Microscope with fluorescence optics equipped with UV- filter set I-3 and photograph was taken.

3.12. *In vivo* studies for plant growth promotion by PSF and PGPR

3.12.1. Mass multiplication and inoculation

3.12.1.1. Mass multiplication of PSF

PSF and BCA isolates were grown separately in the PDA medium for sporulation over a period of 4-5 days after which harvested spore mass (10^6 spores / ml) was suspended in sterile distilled water. For mass multiplication of the PSF, well decomposed FYM heaps were used whereas sand maize meal was used for BCAs. Spore suspension (100 ml) was used to inoculate 5 Kg of FYM. The FYM was first moistened slightly to optimize the PSF growth and kept in polythene bags in shade for 10 days. The mixture was regularly raked every third day during the total of this 10 days period.

Mass culture of fungal pathogens were prepared in sterilized sand maize meal media, in which washed and sterilized sand:water:maize meal ratio of (9:1.5:1; w:w:v) was taken in autoclavable plastic bag and conical flasks (150g) was sterilized at 20 lb for 20 minutes method followed by Biswas and Sen (2000) which was inoculated with mycelial bits of pathogen taken from the margin of actively growing culture and incubated at 28ºC$\pm$ 2ºC for 15-20 days. The two weeks old cultures were used for inoculating the soil. The rhizosphere of each of potted plants was inoculated with 100g of pathogen inoculum prepared in sand maize meal media. Regular watering of the plants was done to assure the successful establishment of the pathogen.

3.12.2. Mass multiplication of PGPR isolates

In case of PGPR isolates, the bacterial isolates were cultured in nutrient broth medium with shaking at 30^o C at 120 rpm for 48 h. At the end of the log phase, bacterial cultures were centrifuged at 3000 rpm for 5 min and the supernatant was discarded. Pellet was scraped into sterile distilled water. The aqueous suspensions were diluted as necessary to maintain the bacterial concentration at 10^6 cells/ml. This

suspension was utilized for seed bacterization of test crops and for direct application in pot and field condition.

3.12.2.1 Application of bacteria

3.12.2.2. Soil drench

The bacteria were grown in NB for 48 h at 28ºC and centrifuged at 2000 rpm for 15 minute. The pellet obtained was suspended in sterile distilled water. The optical density of the suspension was adjusted using UV-VIS spectrophotometer following method to obtain a final density of 3 $X10^6$cfu ml^{-1} .

The bacterial suspension was applied to the pots and rhizosphere of plants in the field conditions. Applications were done @ 0f 100 ml per pot at regular interval.

3.12.2.3. Foliar spray

The bacterial pellet suspended in sterile distilled water at a concentration of $3x10^6$ cfu ml^{-1} after the addition of a few drops of Tween -20 was sprayed until run off on the foliar part of the test crop plants. The spraying was done forth nightly till the new shoots started appearing. The growth parameters such as number of leaves, branches and height were observed.

3.12.2.4. Seed bacterization

Seeds of test plants were surface sterilized with sodium hypochlorite and rinsed in distilled water after which seeds were dried under sterile air stream. Bacterial suspension containing $3x10^6$ cfu ml^{-1} was taken in 500 ml glass beakers. The seeds were soaked in bacterial suspension using 0.2% sterilized carboxymethyl cellulose as an adhesive. The seeds soaked in sterile distilled water served as control. Bacterized seeds were sown in fields in rows supplemented with 0.05% (wt/ wt) Rock phosphate ((RP-140; P=18.8%)) and allowed to germinate. Growth promotions were evaluated after 15 d in terms of increase in root and shoot length and increase in root and shoot biomass in comparison to control. Each treatment was carried out in three replications with at least 50 plants in each replicate under same physical and environmental conditions.

3.12.3. Assessment of plant growth promotion by PSF and PGPR

The experiment was conducted on different types of crop plants which included; legumes (*Cicer aeritenium*, *Glycine max*, *Vigna radiata*, *Pisum sativum* and *Phaseolous vulgaris)* Cereals (*Triricum aestivum* and *Oryza sativa*) as well as a

plantation crop (*Camelia sinensis)*, under greenhouse condition as well as field conditions to assess the efficacy of selected fungal and bacterial isolates to promote plant growth. The growth promotion was assessed in seedling by comparing the increase in height, number of leaves, root length, shoot length, root shoot fresh and dry biomass as well as yield of the treated plants to the untreated control plants under the same environmental and physical condition (temperature 35-30ºC; R.H. 60-80%; 16h photoperiod). The experiments consisted of at least five replicates in each treatment incompletely randomized design.

In case of some of the crops whose seed were bacterized or treated, the plant growth promoting activity were also assessed based on the seedling vigour index, seed germination percentage, root and shoot length of individual seedling to work out the vigour index using the formula suggested by Baki and Anderson (1973). Vigour Index = (mean shoot length = root length) x % germination. The experiment was carried out in three replicates with 30 seeds in each plate.

3.12.4. Assessment of soil phosphate mobilization by PSF and PGPR isolates

3.12.4.1. Modified Morgan Extraction for Phosphorous from soil

Approximately 4cm of air dried, sieved soil of 10 g root or leaf tissue oven dried was put into 50ml extraction flasks. For colourless filtrate, $1cm^3$ of activated carbon (charcoal) was added to each flask. 20 ml of the modified morgan extractant (add 28.74 ml glacial acetic acid as added to a 40L carboxy containing approximately 20L distilled water. And 1825 ml concentrated NH_4OH was added to each flask. Diluted to 40 L with distilled water and mixed well. The pH of the solution was maintained at 4.8± 0.05 by adding concentrated NH_4OH or acetic acid to each flask. It was shaken at 180 oscillations per minute for 15 minutes on reciprocating shaker and finally filtrate was filtered through a medium porosity filter paper. (Ref……)

3.12.4.2. Estimation of Total phosphate content in soil and plant tissues

Quantitative estimation of phosphate was carried out following ammonium molybdate-ascorbic acid method as described by Knudsen and Beegle as described earlier.

3.13. *In vivo* studies of disease suppression by PGPR and BCA

3.13.1. Disease assessment with BCA

Inoculum of Biocontrol agents 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 containing the pathogen (*Thanatophorous cucumeris* and *Sclerotium rolfsii*) infested or control soil (2000 g), 10 g of the wheat bran colonized by the biocontrol agent was mixed to give a concentration of 10^5 cfu / g of soil as described by Chakraborty *et al.* (2006).

In order to determine the effects of biocontrol agents (BCA) on disease reduction, four treatments were taken in each case: 1- Untreated control, 2-Inoculated with pathogen, 3- Inoculation with BCA isolates and 4-Inoculation with both BCA isolate and fungal pathogen. Disease assessment was done after 15 days of inoculation.

The rhizosphere of plants pre- treated with the antagonists or without treatment was inoculated with pathogen. In pre- treated plants, pathogen inoculation was done 3 days after application of antagonist. The inoculated plants were examined after 15 days.

Disease intensity was assessed on the basis of above ground and under ground symptoms. (Roots, colour, rotting, leaves withering, shoot tip withering, defoliation etc.). Percentage of disease incidence was calculated by dividing the number of diseased plants by total number of plants and then multiplying by hundred while disease intensity was calculated by using 0-6 scale as adopted by Mathew and Gupta (1996).

0 = No symptoms;

1 = Small roots turn rotten lesion appeared at the collar region;

2 = Middle leaves start wilting and 10-20% of root turn brown;

3 = Leaves wilted and 20-40% roots become dry with browning of shoot;

4 = Extensive rotting at the collar region of roots, 60-70% of roots and leaves wilted, browning of shoot over 60%;

5 = 80% roots affected while the root along with the leaves withered and shoot becomes brown more than 80%

6 = Whole plants die, since 100% roots were dried.

13.13.2. Disease assessment with PGPR

Ability of PGPR isolates to suppress root diseases of different test crops caused by *Thanatophorous cucumeris* and *Sclerotium rolfsii* were tested in glass house condition in potted plants with 5 plants per pot with three replicates for each treatment. Inoculation of the rhizosphere of test crops with the pathogen and disease assessment was done following the method of Chakraborty *et al.* (2006). For co-inoculations, the PGPR strains to be tested were first applied to the rhizosphere of 15 d old plants as aqueous suspension (10^8 cells/ml) prior to pathogen inoculation and after three days, plants were inoculated with pathogen. The experiment included four treatments: 1- Healthy; 2-Treated with Bacterium but Un-inoculated with the pathogen; 3- Untreated but inoculated with pathogen ; 4- treated inoculated.

Disease assessment was done after 5, 10, 15, 20, 25 and 30 days of inoculation.

Disease index was recorded based on the score 0-6, depending on both underground and above ground symptoms as follows: Root rot index: 0 – no symptoms; 1 – roots and collar region turn brownish and start rotting; 2 – leaves start withering and 20–30% of roots turn brown; 3 – leaves withered and 50% of the roots affected; 4 – shoot tips also starts withering; 60–70% roots affected; 5 – whole plants starts withering ; 6–Whole plant die, with upper withered leaves still remaining attached; roots fully rotted.

13.13.3. Calculation of biocontrol efficacy (BE %) and Disease index (DE %)

For calculating biocontrol efficacy (BE) of both the BCAs and PGPR the disease index was recorded based on the score 0-6, depending on both underground and above ground symptoms.

Disease Incidence and Biocontrol efficiency was calculated as described by Xue *et al*., (2013) using the following formula:

Disease incidence (DI)

$$= \left[\Sigma \text{ (The number of plants in this index} \times \text{Disease index)/ (Total number of plants investigated} \times \text{highest disease index}\right] \times 100\ \%$$

Biocontrol efficacy (BE)

$$= \left[\text{(Disease incidence of control – disease incidence of bacteria treated plants)/}\right.$$

Disease incidence of control] x 100 %

3.14. Assay of defense enzyme activities enhanced after application of BCA and PGPR

3.14.1. β-1, 3-glucanase (β-GLU, EC 3.2.1.38)

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.

3.14.2. Chitinase (CHT, EC 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-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.

3.14.3. Phenyl alanine ammonia Lyase (PAL EC 4.3.1.5)

Extraction of PAL (E.C. 4.3.1.5) was done by following the method described by Chakraborty *et al.* (1993) with modifications. 1gm root and leaf sample was crushed in 0.1M sodium borate buffer pH 8.8 (5ml/gm) with 2mM of ß mercaptoethanol in ice cold temperature. The slurry was Centrifuge in 15000 rpm for 20 minutes at 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.

3.14.4. Peroxidase (POX, EC1.11.1.7)

The reaction mixture contained 1 ml of 0.2M Na-phosphate buffer (pH5.4), 1.7 ml dH_2O, 100µl crude enzyme, 100 µl O-dianisidine (5mg/ml methanol) and 0.1 ml of 4mM H_2O_2. O-dianisidine was used as substrate and activity was assayed spectrophotometrically at 465 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2 (Chakraborty *et al.*, 1993). Specific activity expressed as the increase in Δ A 465/g tissue/min.

3.14.5. Acid and Alkaline phosphatase (EC 3.1.3.2 & EC 3.1.3.1)

About 2 g portions of each soil sample was used for enzyme extraction and assays. The activities of enzymes were expressed according to method of Tominaga and Takeshi (1974) with modifications. For acid phosphatase assay, soil samples were extracted in 5 ml of 50 mM sodium acetate buffer (pH 5.0) using a chilled mortar and pestle which was then transferred into a tube and solution was shaken well. 1 ml of 5 mM p-nitrophenyl phosphate solution was added to the tube. All the tubes along with control were allowed to incubate at 37^{o}C for 1 h. After incubation, 2 ml of solution was transferred into centrifuge tubes. Centrifugation was performed at 3000 rpm for 2 min at 4°C. Finally the supernatant was transferred into clean cuvettes and the reaction was terminated by addition of 4.0 ml of 100 mM NaOH. The amount of p-nitrophenol liberated was determined from the absorbance at 400 nm. Enzyme activity was expressed as mmol p-nitrophenol liberated/sec/g of soil. The procedure for the assay of alkaline phosphatase was similar to acid phosphatase except that for enzyme extraction and incubation 100 mM sodium bicarbonate buffer (pH 10.0) was used.

3.14.6. Estimation of phenols contents

3.14.6.1. Extraction of phenol contents from leaves and roots

Phenols was extracted from the fresh leaves following the method of Mahadeven and Sridar (1982).1 g of fresh root/leaf cut into small pieces put in boiling alcohol in a water bath for 5-10 minutes (4ml alcohol /gm tissue). After 15 minutes of boiling it was cooled and crushed in mortar and pestle thoroughly at room temperature. The extract was passed through two layers of cheese cloth and then filtered through Whatmann No.1 filter paper. Final volume was adjusted with 80% ethanol. The whole experiment was done in dark to prevent light induced degradation of phenol.

3.14.6.2. Estimation of Total phenol

Total phenol content was estimated by Folin Ciocalteau‘s reagent, following the method of Mahadevan and Sridhar (1982). To 1ml of test solution with 10^{-2} and 10^{-1} dilution (leaf and root) plus 1ml of 1N folin –ciocalteau reagent (1:1) followed by 2ml of 20% sodium carbonate solution (Na_2Co_3) is taken in test tube, mix well and boil in water bath for exactly 1minute. After cooling, dilute upto 25ml by adding distilled water. Absorbance of the blue colored solution was measured in a systronic photometric colorimeter Modle 101 at 650 nm. Quantity of total phenol was estimated using caffeic acid as standard.

3.15. 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.

3.15.1. Preparation of genomic DNA extraction buffer

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

Lysis Buffer

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

Genomic DNA Buffer

10 mM Tris, pH 8.0
0.1 mM EDTA
20%SDS

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

3.15.2. Extraction of Fungal Genomic DNA

Isolation of fungal genomic DNA was carried out as outlined by Ma *et al.* 2001. The mycelia (3-4 days old) were incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH8.0), 100 mM NaCl and 2% SDS, for 1 hr at 60^0C followed by centrifugation at 12,000 rpm for 15 min., whereas genomic DNA was extracted from isolates of bacteria and actinomycetes using CTAB buffer. The supernatant was then extracted with equal volume of water saturated phenol and further centrifuged at 12,000 rpm for 10 min; the aqueous phase was further extracted with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 12,000 rpm for 15 min; the aqueous phase was then transferred in a fresh tube and the DNA was precipitated with chilled ethanol (100%). DNA was pelleted by centrifuging at 12000 rpm for 15 min, washed in 70% ethanol and air dried. . Finally the pellets were air dried and suspended in 1X TE buffer and stored at 4^0C until further use.

3.15.3. Extraction of Bacterial Genomic DNA

Isolation of genomic DNA from bacterial DNA was performed by growing the bacterium in 25 ml nutrient broth medium at 30^0C on a shaker. The broth culture was centrifuged at 6000 rpm for 5 min. Pellets were collected and suspended in 0.4 ml of 1X TE buffer (10mM Tris-HCl, pH 8.0 and 1mM EDTA pH 8.0). The suspension was centrifuged again and the pellet was collected and re-suspended in SET buffer (20mM Tris-HCL, 75mM NaCl, 25mM EDTA, pH-8.0) 10µl of Lysozyme was added and incubated for 30-60 min at 37^0C. Next after this initial incubation 10% SDS and 10 µl of Proteinase-K was added and incubated at 55^0C for 60 min. Next 0.3 vol. of 5M NaCl and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and incubated at room temperature with gentle extraction for 30 min. The suspention was then centrifuged at 5000 rpm for 15 min. in a cooling centrifuge, the aqueous phase was removed to a fresh tube. To the clear aqueous phase 0.1 vol of 3M sodium acetate (pH 4.8) was wdded followed by addition of 1 vol of chilled

absolute ethanol and incubated in room temperature for 30 min with gentle extraction. Next the extraction was centrifuged at 10000 rpm for 15 min in a cooling centrifuge and the supernatant was discarded carefully. The pellet was washed in 70% ethanol and centrifuged at 10000 rpm for 10 min in a cooling centrifuge. Finally the pellets were air dried and suspended in 1X TE buffer and stored at 4^0C until further use.

3.15.4. 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.

3.15.5. Spectrophotometric quantification of Genomic DNA

The pure sample is (without significant amounts of contaminants such as a proteins, phenol, agarose, or other nucleic acids), can use spec to measure amount of UV irradiation absorbed by the bases. For quantitating DNA or RNA, readings should be taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample.

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

1 O.D. at 260 nm for RNA molecules = 40 ng/ul of RNA

The reading at 280 nm gives the amount of protein in the sample.

Pure preparations of DNA and RNA have OD_{260}/OD_{280} values of 1.8 to 2.0, respectively. If there is contamination with protein or phenol, this ratio will be significantly less than the values given above, and accurate quantitation of the amount of nucleic acid will not be possible.

3.15.6. 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.

3.15.7. Preparation of DNA samples for electrophoresis

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.

3.15.8. 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.

3.16. RAPD PCR analysis

RAPD analysis of the genomic DNA was conducted using 10 bp long decamerprimer as described by Caetano-Annoles *et al.,* 1991.

For RAPD, random primers were selected (Table-1). Genomic DNA was randomly 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 decamer primer and 1 µl of 50 ng template DNA.

After PCP amplification, 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 by horizontal electrophoresis.

3.16.1. Amplification conditions for RAPD analysis

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

3.16.2. RAPD- PCR primers

The following primers are used in the study.

3.16.3. Analysis of RAPD band patterns

RAPD band patterns were initially assessed by eye and then the image of the gel electrophoresis was documented through Bio-Profil Bio-1D gel documentation system and analysis software.

3.16.4. Scoring of individual bands and construction of dendrogram

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. All reproducible polymorphic bands were scored and analyzed following UPGMA cluster analysis protocol and computed *in silico* into similarity matrix using NTSYSpc-Numerical Taxonomy System Biostastiscs, version 2.11W, (Rohlf, 1993). The SIMQUAL program was used to calculate the Jaccard's coefficients. The result generated in this analysis was then used to generate dendograms using the SHAN clustering programme, selecting the unweighted pair-group methods with arithmetic average (UPGMA) algorithm in NTSYSpc. The isolates were then group isolates with identical band patterns for a given primer. A two (2-D) and three dimensional (3-D) principal component analysis was constructed to group the individuals and test the relationship using EIGEN progamme (NTSYS-PC).

3.17. ITS PCR analysis

All isolates of BCA, PGPR as well as one of the pathogen *T. cucumeris* were taken up for ITS-PCR amplification and was carried out according to the method of Stafford *et al.*, (2005). 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.

3.17.1. ITS-PCR primers

3.17.2. Amplification conditions

Temperature profile, 94^{o}C for 5 min. followed by 30 cycles of denaturation at 94^{o}C for 30 sec, annealing at 58^{o}C (for fungal isolates) 59 ^{o}C (for bacterial isolates) and for 30 sec and extension at 70^{o}C for 2 min and the final extension at 72^{o}C for 7 min in a Primus 96 advanced gradient Thermocycler.

3.17.3. 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. A chromatogram was generated which provided the sequence informations.

3.18. BLAST of Sequence

The DNA sequences were analyzed using the alignment software of BLAST algorithm (http://ingene2.upm.edu.my/Blast, Altschul *et al.*, 1990) for the different characteristic of DNA sequence for the identification of microorganism Identification of microorganism was done on the basis of homology of sequence (http://ncbi.nlm.nih.gov/blast).

3. 19. Submission of rDNA gene to NCBI genbank

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

3.20. 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 Walgorithm (Thompson *et al.*, 1994), included in the Megalign module (DNASTAR Inc.).Multiple alignment parameters used were gap penalty = 10 and gap length penalty = 10. Both of these values are aimed to prevent

Table 1. RAPD and universal ITS primers

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC
RAPD primers				
AA-04	CAGGCCCTTC	10	38.2	70
OPA-4	AATCGGGCTG	10	39.3	60
A-11	AGGGGTCTTG	10	31.8	76
A-5	AGGGGTCTTG	10	36,8	73
OPD6	GGGGTCTTGA	10	32.8	83
OPA1	CAGGCCCTTC	10	38.2	70
ITS-Primers pairs				
Talatomyces flavus and *Thanatophorous cucumeris*				
T/ITS-1	TCTGTAGGTGAACCTGCGG	19	63.9	57
T/ITS-4	TCCTCCGCTTATTGATATGC	20	61.5	45
Trichoderma isolates				
T/ITS 1	TCTGTAGGTGAACCTGCGG	19	63.9	57
T/ITS4	TCCTCCGCTTATTGATATGC	20	61.5	45
Bacterial 16S rDNA Universal primers				
16Srrna	AGAGTRTGATCMTYGCTWA*	19	54.5	42
16S rrna	CGYTAMCTTWTTACGRCT	18	58.5	40

* Degenerate universal primer for 16Sr RNA gene

lengthy or excessive numbers of gaps. The default parameters were used for the pairwise alignment. The use of Clustal W determines that, once a gap is inserted, it can only be removed by editing. Therefore, final alignment adjustments were made manually in order to remove artificial gaps. Phylogenetic analyses were completed using the MEGA package (version 4.01; Institute of Molecular Evolutionary Genetics, University Park, PA). Neither gaps (due to insertion-deletion events) nor

equivocal sites were considered phylogenetically informative. Hence, complete deletion prevented the use of any of these sites in further analyses. Phylogenetic inference was performed by the UPGMA method (Sneath and Sokal, 1973). Bootstrap tests with 1,000 replications were conducted to examine the reliability of the interior branches and the validity of the trees obtained. There were a total of 138 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4 as described by Tamura *et. al*., 2007.

3.21. Analysis of rDNA region for DNA molecular weight, nucleotide frequency and ORF.

Combinations and percentage of occurrence of different nucleotide in the entire sequence was calculated using the bioinformatics algorithm from the website- www.ualberta.ca/~stothard/ javascript/dna_stats.html. The DNA Molecular weight of rDNA sequences was calculated with the help of DNA weight calculator (www.ualberta.ca/~stothard/javascript/dna_mw.html). Similarly number of ORFs in the given sequence was calculated with the help of online bioinformatics tool from- www.ualberta.ca/~stothard/javascript/orf_find.html and www.star.mit.edu/orf/runapp_html.html.

3.22. Denaturing Gradient Gel Electrophoresis (DGGE)

3.22.1. PCR amplification of gnomic DNA of the isolates for DGGE analysis

Denaturing Gradient Gel electrophoresiswas performed according to the method of (Zhao *et al.,* 2006). 18S/16S DNA (200 bp with GC clamp) was amplified with the forward primer containing GC clamp at 5' end (F352T: 5'- CGC_CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG TGG C-3' and 519r: 5'-ACC GCG GCT GCT GGC AC- 3') in 25 µl of reaction mixture containing 1×PCR buffer, 2.5mM $MgCl_2$ (Bangalore Genei, India), 100 ng of the template DNA, 25.0 pmol each of the forward and reverse primers, 250 µM each of dNTPs, and 1 U of *Taq* DNA polymerase (Bangalore Genei, India). The touchdown PCR program was performed which consisted of an initial denaturation at 95^{o}C for 5 min, followed by 6 cycles of 95^{o}C for 1 min, 65^{o}C for 1 min, and 72^{o}C for 1 min, in which the annealing temperature was reduced by 0.5^{o}C/cycle from the preceding cycle, and then 24 cycles of 95^{o}C. Perpendicular DGGE was performed with "The Decode Universal Mutation Detection System" (Bio-Rad Laboratories, USA). A uniform gradient gel of 0% to 100% denaturant was prepared which was changed

several times so as to optimize suitable concentration and finally 20 to 60% denaturant was found optimal for the best result.

3.22.2. Denature Gradient Gel Electrophoresis of the PCR products

3.22.2.1. Reagents and solutions required for DGGE analysis

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

50 x DGGE/TAE buffer solution

Trizma-Base:	484.4 grams
Sodium-Acetate:	272.0 grams
trisodium EDTA	37.2 grams
H_20	2 liters

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

Preparation of Denaturants

100% Denaturant:	
Urea	42.0 grams
38.5% Acrylamide (makes a 6.5% gel)	16.9 ml
50x DGGE/TAE	2.0 ml
Formamide	40.0 ml
Filled up to 100 ml with distilled H_2O.	
0% Denaturant	
38.5% Acrylamide	16.9 ml
50x DGGE/TAE	2.0 ml
Ammonium Persulphate	10% (w/v)
TEMED	20µl

3.21.2.2. Creating the gel sandwich (DCode System BioRad)

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

3.22.2.3. Preparing the gel

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

UF solution	[UF](%)	[Acrylamid/Bis] (%)	Volume UF solution (ml)	Volume APS 10% (µl)	Volume TEMED (µl)
Low	30-45	6	13	78	6
High	60	6	13	78	6

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

3.22.2.4. Running a gel

Fresh 0.5x TAE buffer was added to the buffer tank to the mark "Fill". The DCode™ Universal Mutation Detection System (Bio-Rad) was switched on at least 60 minutes before electrophoresis, so that the buffer can heat up to 60°C. After 2-3 hours of polymerization, the comb was removed carefully and the bottom of the sandwich was rinsed with tap water to remove non-polymerized gel .The sandwich was set in the sandwich-holder. A dummy sandwich was also set at the other side to get a closed upper buffer compartment. (A dummy consists of a large and small glass plate stuck together with no spacers in between). The DCode™ was then switched off and the lid taken off after 1 minute. The sandwich holder was slided into the buffer tank, with the red dot of the cathode at the right side. The DCode™ pump and the stirrer underneath the tank were switched on (300 rpm) until samples were loaded.

3.22.2.5. Staining of gels and photography

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

5 minutes and distained with running buffer. The gel was photograph under UV transilluminator.

3.22.2.6. Data analysis.

3.22.2.6.1. Scoring of individual bands

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

3.22.2.6.2. UPGMA analysis of the DGGE bands

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