### 3.1 Soil samples

In order to isolate rhizosphere microorganisms, soil samples were collected from both the hills and plains. In hills, Margaret's Hope Tea Estate, Darjeeling (2000 meters above sea level) was selected and in the plains 6 sites of Dooars and Terai were selected as follows:

**Table 1:** Sources of rhizosphere soil for isolation of microorganisms

| Name of estate | Age of bushes(years) |
|---|---|
| Hansqua Tea Estate, Terai | 90 |
| Kadommini Tea Estate, Dooars | 50 |
| CoochBehar Tea Estate, Dooars | 70 |
| Chandmoni Tea Estate, Terai | 80 |
| Tea Research station, Nagrakata, Dooars | 40 |
| Tea Experimental Garden, NBU | 10 |

Collected soil samples were used for determining microbial population and isolation of microorganisms.

### 3.2. Isolation of microorganisms from soil

Rhizosphere soils of healthy tea plants were chosen for the determining microbial population and isolation of microorganisms. In each case, 5gm of soil particles loosely adhering to the roots were collected. The soil suspension was made by dissolving the soil sample in 30 ml of sterile distilled water using magnetic stirrer for 1 h. The suspension was allowed to settle down till the two distinct layers were clearly visible. Then the upper light brown colored layer was pippetted out and serial dilutions were prepared. 1ml of each of $10^{-3}$ and $10^{-5}$ dilutions was actually used for isolation by dilution plate technique (Kobayashi *et al.*, 2000) using Nutrient Agar (NA- peptone 5 g, NaCl 5 g, beef extract 1.5 g, yeast extract 1.5 g, agar 12 g and distilled water 1 L; pH 7.2± 0.2), King's B media (KB- peptone 20 g, $K_2HPO4$, anhydrous 1.5g, $MgSO_4.7H_2O$ 1.5 g, Glycerol 15 ml in 1 L distilled water, pH 7.4± 0.2) and Potato Dextrose Agar (PDA-potato 400g, dextrose 20 g, agar 20 g, distilled water 1 L; pH 7.3± 0.2) as the growth media. The petriplates were then incubated for 1-5days at 28±2$^0$C for the observation of microbial growth.

### 3.3. *In vitro* testing for antagonism to pathogens

#### 3.3.1. Solid medium

To know the antagonistic activity of individual bacterial strain against root fungal pathogens, *in vitro* tests were done in dual culture using PDA or NA. Agar blocks (6mm) containing 7 day old mycelia of fungi were placed 3.5cm apart on the medium in a petridish. Each bacterial isolate was streaked at one side of the agar plate about 1 cm away from the edge or by placing the fungal inocula in the centre and streaking the bacteria around the fungal inocula. For each test three replicate plates were used. The plates were inoculated for 5-7 days at 28±2$^0$C and inhibition zone towards the fungal colony in individual plate was quantified. Results were expressed as mean of percentage of inhibition of the growth of the pathogen ± standard error in presence of the bacterial isolates.

#### 3.3.2. Liquid medium

To assess the possible mutualistic antagonisms between the fungus and bacteria in mixed culture method outlined by Chakraborty and Chakraborty (1989) was followed. PDB and NB were selected because it allows vigorous growth of both the fungus and bacteria. One agar block (6mm dia.) containing 7 day old mycelia of the fungus and 0.5 ml of bacterial suspension (1x10$^6$ cfu/ml) were used as inocula for each flask (50 ml NB/250 ml flask). The mycelium grown without the bacterial strain in the similar medium was taken as the control sample. The cultures were inoculated at 28$^0$±2$^0$C and after 15 days of incubation mycelia were washed thoroughly with sterile distilled water to remove bacteria as far as possible and harvested by staining through muslin cloth and mycelial dry weights were determined

### 3.4. Selection and identification of antagonistic microorganisms

#### 3.4.1. Microscopic observation

##### 3.4.1.1. Fungi

The isolated fungi were allowed to grow in Petridishes (7cm) containing sterile PDA medium for 7 days, then nature of mycelia growth, rate of growth and time of sporulation were observed. For identification, spore suspensions were prepared from individual culture. Drops of spore suspensions were placed on clean, grease free glass slides, mounted with lactophenol-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.

### 3.4.1.2. Bacteria

Gram staining technique was followed for microscopic identification of rhizobacterial cultures. The slide was placed on a rack and flooded the entire slide with crystal violet and allowed the crystal violet to stand for about a minute. When the time had elapsed, the slide was washed with tap water for 5 seconds. Next, the slide was flooded with the iodine solution and allowed it to stands for a minute as well. After a minute the slide was rinsed with tap water for 5 seconds, after which ethanol was added. The ethanol was added drop wise until no blue-violet color was emitted from the slide. Finally the slide was rinsed with water for 5 s, dried and observed under microscope.

### 3.4.2. Biochemical tests

#### i. Endospore stain

A bacterial smear was prepared on the slide and passed over the flame for fixing. The fixed slide was flooded with the solution of malachite green and placed over boiling water bath for 5 min. After rinsing, the smear was counter stained with safranin.

#### ii. Fermentation of Carbohydrates

Three test tubes containing 0.5% solution of dextrose, sucrose or lactose, nutrient broth, and a Durham tube were inoculated with the unknown isolate and incubated at room temperature for 24 h. Yellow color indicates acid production and air in the Durham indicates gas production.

#### iii. Voges Proskuser reaction

Culture tubes containing 10 ml sterile VP broth were inoculated with the bacterial isolates and incubated at $37^0$C for 3days.To the cultures 0.6 ml of 5% w/v α-ethanol-napthol and 0.2 ml of 40 % w/v aqueous potassium hydroxide were added and kept at room temperature for 1 h for production of pink color indicating positive reaction.

**iv. Catalase**

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

**v. Urea digestion**

A test tube containing nutrient broth, urea, and phenol red was inoculated with the unknown isolate and incubated at room temperature for 24 h. A fuchsia color indicates the digestion of urea.

**vi. Esculine hydrolysis**

The slants containing esculine hydrolysis test medium were inoculated with 24 h old bacterial culture and incubated at $30^0$C for 7 days. Blackening of the medium indicates the hydrolysis of esculine.

**vii. Casein hydrolysis**

The milk agar was streaked with the bacteria and was incubated at $37^0$C and observed for the clear zone around the streaks.

**viii. Starch hydrolysis**

The bacteria were streaked on sterilized starch agar plates (NA + 0.1 % soluble starch) and inoculated for 5 days at $37^0$C. The plates were flooded with Lugol's Iodine solution. The clear zone underneath and around the growth indicates starch hydrolysis.

**ix. Indole test**

10 ml of Davis Mingoli's broth supplemented with 0.1 % tryptophan was inoculated with the isolates and incubated an aerobically at $37^0$C for 7 days. The culture were layered carefully with 2 ml of Ehrlich-Bohme (p-dimethyaminobenzaldhyde 10 g, concentrated HCL 100 ml ) reagent on the surface, allowed to stand for a few minutes and observed for the formation of a ring at the medium reagent interface indicating the production of indole.

**x. Reduction of nitrate to nitrite**

Sterile nitrate broth was inoculated with test isolates and incubated at $37^0$C till the medium became turbid. Three drops of nitrate reduction test reagent (Sol. A: $H_2SO_4$ 0.8 g, 100 ml 5 N Acetic acid; Sol. B: 0.5 napthylamine and 100 ml acetic acid. The solutions A and B were mixed in equal volume just before use) was mixed

in 1 ml of the cultures and observed for the development of red or yellow color indicating the presence of nitrite.

**xi. Gelatin liquefaction**

The organism was inoculated (stabbed) into a solid medium containing gelatin; liquefaction at room temperature or inability to resolidify at refrigerator temperature indicates the presence of photolytic enzymes.

**xii. $H_2S$ production**

Organism was inoculated into peptone iron medium; formation of black sulfides indicates that organism produces hydrogen sulfide.

**xiii. Citrate utilization**

Organism was inoculated into citrate agar medium in which citrate is the sole carbon source; an indicator in the medium changes color if citrate is metabolized; use of citrate indicates the presence of the permease that transports citrate into the cell

### 3.5. Test organisms

#### 3.5.1. Fungal Culture

For *in vitro* evaluation of antagonistic activity of rhizobacterial isolates, following root fungal pathogens viz., *Poria hypobrunnea, Fomes lamaoensis, Sphaerostilbe repens, Ustulina zonata, Sclerotinia sclerotiorum* and *Sclerotium rolfsii* were used.

#### 3.5.2. Source of culture

Tea roots pathogen *Poria hypobrunnea* Petch. was obtained from UPASI Tea Research station, Valparai, Tamil Nadu. The cultures are being maintained in the laboratory with regular sub culturing in PDA for subsequent test. Other pathogens used for the experimental purposes are mentioned in Table 2. *Sclerotium rolfsii* I Sacc. causing seeding blight in tea, *Sclerotium rolfsii* 11 Sacc. causing crown rot in orchid and collar and root rot disease in *Cicer arietinum* and *Sclerotinia sclerotiorum* (lib.) de Bary causing white rot, isolated from *Vanda roxburgii* were obtained from Immuno-Phytopathological laboratory, Department of Botany, North Bengal University .

**Table 2:** List of fungal culture used in investigations.

| Species | Host | Source |
|---|---|---|
| *Poria hypobrunnea* | Tea | UPASI Tea Research station, Valparai |
| *Fomes lamaoensis* | Tea | Tea Experimental Station, Tocklai, Assam |
| *Armillaria mellea* | Tea | UPASI Tea Research station, Valparai |
| *Rosellinia arcuata* | Tea | UPASI Tea Research station, Valparai |
| *Sphaerostilbe repens* | Tea | Tea Experimental Station, Tocklai, Assam |
| *Ustulina zonata* | Tea | Tea Experimental Station, Tocklai, Assam |
| *Sclerotium rolfsii*- i | Tea<br>Soybean | Immuno-Phytopathologicallaboratory,<br>Department of Botany, N.B.U |
| *Sclerotium rolfsii*- ii | Pea<br>Vanda | Immuno-Phytopathologicallaboratory<br>Department of Botany, N.B.U |
| *Sclerotinia sclerotiorum* | Vanda | Immuno-Phytopathologicallaboratory<br>Department of Botany, N.B.U |
| *Alternaria alternata* | Tea<br>Soil | Immuno-Phytopathologicallaboratory<br>Department of Botany, N.B.U |
| *Fusarium oxysporum* | Soil | Immuno-Phytopathologicallaboratory, NBU. |

### 3.6. 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.

#### 3.6.1. Solid media

To assess the mycelial growth of *Poria hypobrunnea* in solid media, the fungus was first grown on petridishes, each containing 20 ml of PDA and incubated for 5-8 days at 30±2$^0$C. Agar block (6 mm diameter) containing the mycelia was cut with sterile cork borer from the advancing zone of mycelial mat and transferred to each petridish containing 20 ml of sterilized solid media. Finally diameter of mycelia was measured at regular interval of time. The media were as follows: Potato Dextrose Agar, PDA (Peeled potato-40.00g, Dextrose-2.00g, Agar- 2.00g, Distilled water-100ml); Richard's Agar, RA ($KNO_3$- 1.00g, $KH_2PO_4$- 0.50g, $MgSO_4$, $7H_20$ 0.25g Sucrose- 3.00g, Agar-1.50g, Distilled Water-100ml); Elliot's Agar, EA ($KH_2PO_4$-0.136g, $Na_2CO_3$-0.106g, $MgSO_4$, $7H_2O$-0.050g, Dextrose-0.500g, Asparagine-0.100g, Agar-1.50g), Carrot Juice Agar, CJA (Grated carrot-20.00g, Agar-2.00g, Water-100ml), Potato Sucrose Agar, PSA (Peeled potato-40.00g, Sucrose 2.00g, Agar-2.00g,

Water-100ml); Czapek-Dox Agar, CDA ($NaNO_3$ .0.20g, $K_2HPO_4$ .0.10g, KCl-0.05g , $FeSO_4$, $7H_20$-0.05g, Sucrose-3.00g, Agar-2.00g, Water-100ml); Yeast Dextrose Agar, YDA (Yeast extract-0.75g, Dextrose-2.00g, Agar-1.50g, Agar-1.50g)

### 3.6.2. Liquid media

To assess the mycelial growth of *P. hypobrunnea* in liquid media, the fungus was first grown on petridishes, each containing 20 ml of PDA and incubated for 5-8 days at $30^0 \pm 2^0$C. From the advancing zone, mycelial block (6mm) was cut with sterilized cork borer and transferred to Erlenmeyer flask (250ml) containing 50 ml of sterilized liquid media (NB or PDB) and incubated for 6-8 days with constant stirring at room temperature. After incubation the mycelia were strained through muslin cloth, collected in aluminum foil cup of known weight and dried at $60^0$C for 96h. cooled in desiccators and weighed.

## 3.7. Assessment of bacterial growth

For assessment of bacterial growth in liquid medium, 1 ml of bacterial suspension was inoculated into the medium and allowed to grow for desired period. Following growth, absorbance was noted in a colorimeter at 600 nm. Absorbance was counted into cfu/ml from a standard where known concentration of bacterial suspension was used. The cfu values were counted to log whenever needed. For assessment of bacterial growth, different media were used. These were Potato Dextrose Broth, PDBA (Potato 400g, Dextrose 20g, Distilled $H_20$ 1L, pH 7.3 ± 0.2); Nutrient Broth, NB (Peptone 5.0g, NaCl 5.0g, Beef extract 1.5g, Yeast extract 1.5g, Distilled $H_20$ 1L, pH 7.4 ± 0.2); King's B, KB (Peptone 20.0g, $K_2HPO_4$ 1.5g, $MgSO_4.7H_20$ 1.5g Glycerol 15 ml, Distilled $H_20$ 1L, pH 7.4 ± 0.2); Luria Broth, LB (Peptone 10.0g, NaCl 5.0g, Yeast extract 1.5g, Distilled $H_20$ 1L, pH 7.2 ± 0.2); Nutrient Sucrose Broth, NSB (Sucrose 1.5 g, Yeast extract 1.2 g, Peptone 1.2 g, Beef extract 0.6g, Distilled $H_20$ 300ml, pH 7.2 ± 0.2); Glucose Yeast Peptone, GYP (Glucose 6.0 g, Peptone 3.0g, Yeast extract 1.5g, Distilled $H_20$ 300 ml, pH 7.2 ± 0.2)

## 3.8 Plant materials

### 3.8.1. Tea

#### 3.8.1.1. Selection

Tea clones were originally collected from different geographical locations of India: (a) Darjeeling Tea Research Centre, Darjeeling, (b) Tea Experimental Station,

Tocklai, Assam and (c) Tea Research station, United Planters Association of South India (UPASI), Tamil Nadu and maintained in the Germ Plasam Bank at Department of Botany, North Bengal University. For present investigation following tea varieties were used for the experimental purposes. Darjeeling varieties (K1/1, CP1, S449 and HV 39), Tocklai varieties (TV 20, T 78, TV 18 and T 17) and UPASI varieties (UP 26, UP 3 and BSS 2)

**3.8.1.2. Propagation of Tea**

Tea plants were propagated by cutting method or by seeds. In case of propagation by cuttings, plants were raised by planting cuttings made from shoots of the mother bushes. The standard form of a tea cutting is a piece of stem 2.5cm to 3.5 cm long with a good mature leaf Soil preparation is the most important in propagation technique and hence, care was taken to prepare the soil well. Sandy soil (75 % sand and 25 % soil) with a pH 4.5-4.9 was used. Soil pH was adjusted by applying 2 % aluminum sulphate.

Polyethylene sleeves (8" x 6") were filled up with the prepared soil and stacked in bed and watered thoroughly. The cuttings of all varieties were allowed for rooting in individual sleeves after dipping them in rooting hormone. Sleeves of each bed were covered with polythene cloche and the whole setup was kept under a green agro house. (Plate 2)

In case of young seedling, manuring (aluminum phosphate-8 parts by weight, ammonium phosphate- sulphate 16:20-35 parts by weight) was done after rooting following method of Ranganathan and Natesan (1987). The mixture was dissolved @ 30 g in 1 L of water and applied @ 50 ml per plant. Tipping was done once in a year to promote lateral branching in young plants but in case of mature plants two year of deep pruning cycle was maintained.

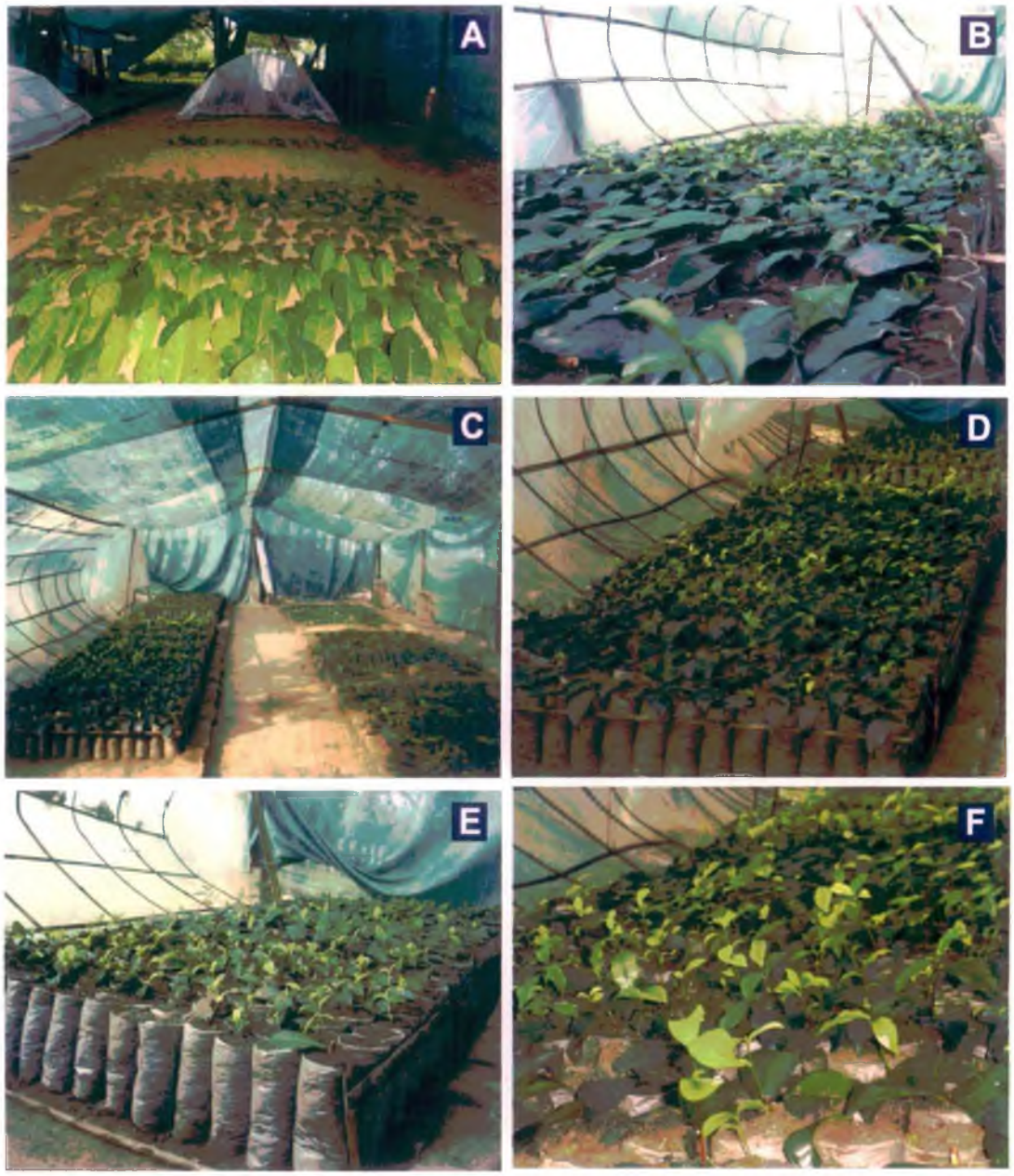


**Plate II (A-F):** Stages of Tea propagation by cutting in Nursery.

### 3.8.1.3. Plantation

For the plantation of tea varieties in the experimental plots pits were prepared for each plant. Before that, simazine @ 75 g / 20 liter water and glyphosphate @ 1:200 were used in the experimental plots for the suppression of weeds. Then pits (1 ½' X 1 ½' X 1 ½') were dug at the intervals of 2' between plant and 3.5' between rows to row. Planting mixture was prepared in the ratio 4.5 kg of well rotten dry cattle manure, 30 kg rock phosphate, 30 kg super phosphate and 2.5 g phorate ( O,O-diethyl S-( ethylthiomethyl ) phosphate dithioate ).At the bottom of the pit, little bit of rock phosphate was placed following which half portion was covered with cattle manure soil mixture. Phorate was mixed with a portion of excavated soil and applied approximately 5cm below the ground level.

Following soil conditioning, plants were inspected, selected and brought to the experimental garden and planted in the prepared soil and pits were refilled upto ground level with conditioned soil. For experimental purposes tea plants of all the varieties were also grown in earthen pots (one plant/pot, 30cm dia.) each containing 5kg soil mixture 1:1). Ten month seedling with well developed shoot and root system were transferred from the sleeves to the pots. These were then maintained in the pots with regular watering.

### 3.8.2. Chickpea

The seeds of chickpea (*Cicer arietinum*) viz. ICCV2 (Swetha) required for experimental purpose were obtained from the seed germplasm bank of International crop Research Institute for Semi Arids and Tropics, Patancheru, Andhra Pradesh. Seedlings were then grown in the Experimental garden of Botany Department, North Bengal University.

### 3.8.3. Mungbean

The seeds of mungbean (*Vigna radiata* L.) viz. Pusa Baishakhi was collected from Pulses and Oils Seed Research Station, Berhampore, West Bengal.

### 3.8.4. Marigold

Seedlings of Marigold (*Tagetes erecta* L.) were collected from the local market and maintained in the Experimental garden of Immuno-PhytoPathology Lab. Department of Botany, North Bengal University.

## 3.9. *In vitro* characterization of PGPR activity of selected bacteria

### 3.9.1. IAA production

To determine the production of IAA, the selected bacterial cells were grown for 24h to 48h C/N ratio medium. Tryptophane (0.1mM) was added in order to enhance Indole acetic acid (IAA) production by the specific 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 liter in 7.9M $H_2SO_4$ 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

### 3.9.2. Phosphate solubilisation

To assess the phosphate solubilization potential of selected bacterial Pikovskaya's agar medium (Pikovskaya, 1984) was used. Bacterial strains were individually spot inoculated at center of the agar plates followed by incubation at $30\pm2^0$C for 5 – 6 days. Formation of clear zone around the bacteria is an indication of the phosphate solubilising capacity.

### 3.9.3 Siderophore production

Production of siderophore was detected following standard method (Schwyn and Neiland, 1987) using blue indicator dye chrome azurol S (CAS). For production CAS agar, 1 L, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1mM $FeCL_3$. $6H_20$ in 10 mM HCl).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 bacteria were spot inoculated at the center of the plates and incubated for 12-15 days. The change in the colour of the medium around the bacterial spot is an indication of siderophore production.

The bacterial strains were streaked separately into the Petriplates containing PDA amended with $FeCl_3$ at concentration of 150µg $ml^{-1}$, 300µg $ml^{-1}$ and 600µg $ml^{-1}$.The actively growing discs of fungal test pathogen *P .hyprobrunnea* was placed

opposite the bacterial streak simultaneously and plates were incubated at 30±2$^0$C for 7 days. Then zone of inhibition around the bacteria was measured. The plate containing the same medium but without $FeCl_3$ was treated as control. The experiment was done in replicates of three.

### 3.9.4 HCN production

Method as described by Wei *et al.* (1991) was followed with slight modification for determination of HCN production by selected bacterias. Bacteria were grown on NA medium supplemented with glycine (4.4 g $L^{-1}$) in a Petri plate. A strip of sterilized filter paper saturated with a solution containing picric acid 0.5% and sodium carbonate 2% placed on upper lid of petriplate. The plates were sealed with paraflim and incubated for 2-4 days. HCN production was indicated by the changes in color of the filter paper strip from yellow to brown to red.

### 3.9.5. Chitinase production

Secretion of chitinase by the selected bacteria was determined following conventional plate method using chitinase detection agar (CDA). Plates were prepared by mixing 10 g colloidal chitin with 20 g of agar in M9 medium ($Na_2HPO_4$ 0.65 g $KH_2PO_4$ 1.50 g NaCl 0.25 g, $NH_4Cl$ 0.50 g $MgSO_4$ 0.12 g, $CaCl_2$ 0.005 g and distilled water 1 L; pH 6.5).The CDA plate was spot inoculated with organism followed by incubation at 30±2$^0$C for 7-8 days. Formation of clear zone around the bacteria was considered positive reaction (West and Colwell, 1994). The colloidal chitin was prepared by following the method described by Roberts and Selitrennkoff (1988), 5 g of chitin powder was slowly added to 60 ml of concentrated HCl and left at 4$^0$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$^0$C.The precipitation formed was collected by centrifugation at 7,000 rpm for 20 min at 4$^0$C and washed with sterile distilled water until the colloidal solution became neutral (pH 7.0). The prepared colloidal chitin solution (5 %) was stored at 4$^0$C until further use.

### 3.9.6. Volatile production

The antagonist and pathogen were inoculated in different Petri plates on PDA. Petri plate containing pathogen was inverted over the Petri plate-containing antagonist and sealed with adhesive tape (paraflim) keeping antagonist in lower and pathogen in

upper Petri plate (Dennis and Webster, 1971). Four sets of experiments were conducted. In one ste, the antagonists and pathogens of the same age were taken. In the second set, the antagonist culture was one day older than pathogen; in the third, the antagonists were 2 days old and in the fourth, the antagonists were 3 days old than pathogen. Each plate was incubated at 28±2$^0$C for 4 days. Radial growth of pathogen was measured after definite time interval. The plate without antagonist served as control. Each experiment considering a single bacterial isolate was performed in triplicate.

### 3.10. Application of bacteria

#### 3.10.1. Soil drench

The bacteria were grown in Nutrient Broth for 3 days at 30$^0$±2$^0$C with constant shaking. Then the bacteria culture centrifuged at 12,000 rpm for 15 min. and the pellet were collected and suspended in sterile distilled water. The optical density of the suspension was adjusted using a UV-VIS spectrophotometer following method to obtain a final density of $3x10^6$ cfu $ml^{-1}$

The bacterial suspension was applied to the rhizosphere of potted plants either singly or in combination during transplantation of seedling from sleeves. Application was done @ 100 ml per pot at regular interval of one month for three months subsequently. The rhizosphere of two years old potted plants were inoculated twice at an interval of 20-25 days.

#### 3.10.2. Foliar spray

The bacterial suspension was also applied to the foliar parts of tea bushes. After addition of few drops of Tween-20, bacterial suspension was sprayed to the pruned bushes of tea to observe the shooting patterns. The spraying was done every fortnight till the new shoots started appearing. The growth parameters such as number of leaves, branches and height were observed.

#### 3.10.3. Seed bacterization

Seeds were surface sterilized with sodium hypochlorite and rinsed in distilled water after which seeds were dried under sterile air stream. 10ml of bacterial suspension containing 3 x $10^6$ cfu $ml^{-1}$ was taken in Petri plates. To this, 100mg of carboxymethyl cellulose was added as adhesive material. The seeds soaked in sterile

distilled water served as control. After 24 h the seeds were blotted dry, and sown in plastic pots (6" dia). Following germination, 10-12 plants were maintained in each pot.

### 3.11. Inoculation techniques

#### 3.11.1 Preparation of pathogen inocula

The inocula of *P. hypobrunnea* were prepared in media of sand maize meal, tea root pieces and tea waste. Following preparations were used for experimental purpose.

(i) Sand maize meal media (SMM): The medium was prepared following the method described by Biswas and Sen (2000). SMM medium (maize meal: washed sterilized sand: water = 1:9:1.5, w: w: v) in autoclavable plastic bag (150g) was sterilized at 20 lb. for 20 min.

(ii) Tea root pieces: Inocula in tea root pieces was prepared following the method of Hazarika *et al.* (2000). First root and root lets were cut into small pieces (3cm x1.5cm x 1.5cm). Root pieces (22-25) were taken in 250ml flask and pieces were dipped in water for 2h and autoclaved as described above. On the next day water was decanted in a sterile chamber and root pieces were inoculated with *P. hypobrunnea*

In another method (Dhingra and Sinclair, 1985) the root pieces were boiled for 30min and sterilized in same way.12-15 sterilized root pieces were then transferred in Erlenmeyer flask (250ml) containing 20 days old *P. hypobrunnea* culture on PDB.

(iii) SMM + root pieces: In this medium SMM along with 6-8 root pieces were sterilized.

(iv) Tea waste: In this medium tea waste and sand were mixed in ratio 1:1, wetted with water and sterilized in polythene packet.

In all cases media were inoculated with *P. hypobrunnea* and incubated for 15-20 days $30^0 \pm 2^0$C.

#### 3.11.2 Inoculation of healthy tea plants

##### 3.11.2.1. Pot grown plants

In case of potted plant, 2-3 year old potted plants were used. Then 100g of *P. hypobrunnea* inoculums were added carefully in the rhizosphere and ensured that

inocula were attached to healthy tea roots. Disease assessment was done after 15, 30and 45 days of inoculation.

**3.11.2.2. Field grown plants**

Tea plants of desired ages from different variety to be tested were selected from plots in the Experimental Garden. The inoculation technique was same as described for potted plants except that 200g inocula were added in each pit. Different combinations were set with PGPRs and after inoculation maintenance was done with regularly watering.

**3.12. Disease assessment**

In order to determine the effect of bacterial isolates on disease reduction, four treatments were taken in each case

i) Untreated control.

ii) Inoculation with pathogen

iii) Inoculation with bacterial isolates.

iv) Inoculation with both bacteria and fungal pathogen.

Initially the rhizosphere was drenched with bacterial suspension prepared in sterilized water two days prior to pathogen application. *P. hypobrunnea* grown in sand maize meal medium were used as fungal inocula. Disease assessment was done after definite time intervals. The 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 0-5 scale as adopted by Mathew and Gupta (1996).

The disease infection observation were recorded in a continuous 0-6 scale, where 0= no symptoms; 1= small roots turn brownish and start rotting; 2 = leaves star withering and 20-30 % of root turns brown; 3 = leaves withered and 50 % of leaves affected; 4 = shoot tips also start withering and 60- 70 % root affected; 5 = shoot withered with defoliation of lower withered leaves and 80 % roots affected; 6 = whole plant die with upper withered leaves still remaining attached and roots fully rotted.

### 3.13. Determination of plant growth promoting activity

#### 3.13.1. Tea

Determination of plant growth promoting activity by the bacteria was assessed under green house condition. The growth parameters such as increase in height, number of leaves and shoot were observed in treated plants in relation to the untreated control plants under the same environmental and physical condition (temperature 30-34$^0$C; R.H. 60-80 %; 16 h photoperiod). The experiment consisted of tea replicates in each treatment in completely randomized design. Observations were recorded after 2 and 4 months of final application of bacterium. The growth promotion by foliar application of bacterial suspension was assessed by the time of appearance of new shoots, overall growth of canopy and general increase in biomass.

#### 3.13.2. Chickpea and Mungbean

The plant growth promoting activity of bacteria in chickpea and mungbean were assessed based on the seedling vigour index by standard roll towel method. Percentage of seed germination was calculated then the root and shoot length of individual seedlings was measured to work out the vigour index. The vigour index was calculated by using the formula by Baki and Anderson (1973):

Vigour Index = (mean root length + mean shoot length) x germination percentage. The experiment was carried out in three replicates with 30 seeds in each plate

#### 3.13.3. Marigold

In case of Marigold, plant growth promotion was assessed on the basis of increase in height and no. of flowers after application of bacterial suspension as a soil drench to the rhizosphere of 2 month old plant.

### 3.14. Extraction of antifungal compounds from rhizobacteria

#### 3.14.1. Cell free culture filtrate

To prepare bacterial culture filtrate loopful of bacteria from a 24h old Nutrient Agar slant were inoculated into Nutrient Broth (50ml/250 flask ) and incubated at 35±2$^0$C with vigorous shaking on a platform shaker at 175 rpm for 96h.Centrifugation was done at 12000 rpm for 20 min. and supernatant was sterilized by cold and hot sterilization. Cold-sterilization was done by passing through sterilized micro filter (

0.22 μm pore size ).Heat-killed culture filtrate was prepared by autoclaving the supernatant at 15 lb pressure for 15 min.

### 3.14.2 Solvent extraction

In order to isolate the active principle from the culture filtrates, the bacteria were grown in NB for 96h at 35±2$^0$C in shaking condition at 175 rpm. Centrifugation was done at 12000 rpm for 20 min. and supernatant was pooled and were used as the culture filtrates. Cell-free sterilized culture filtrate was extracted separately three times with equal volume of acetone, benzene, chloroform, ethylacetate and diethyl ether The organic fraction and corresponding aqueous fraction were evaporated to complete dryness in a rotary evaporator at room temperature and residue in each case was dissolved in 2.5 ml of 80 % methanol and stored in a capped bottle at 4$^0$C and used for the bioassays.

### 3.14.3. Bacterial cell

Beside solvent extraction, antifungal compounds were also extracted directly from the bacterial cells. Extraction was done with slight modification of the method described by Howell and Stiponovic (1980). The bacterial lawn was prepared in NA plate for 7 days. The agar plates were cut into 1cm squares and extracted with 200ml of 80% aqueous acetone, filtered through cheese cloth and the filtrate was centrifuged at 12,000rpm for 10 min. The supernatant was evaporated to remove acetone. To residue (5g per 100ml), NaCl was added and centrifuged at 12,000rpm for 10 min. Each portion of supernatant was extracted twice in diethyl ether and dried over anhydrous calcium chloride. The compound in a crystalline form was removed from this residue. This was recrystalized by dissolving in methanol for further studies

## 3.15. Bioassay of active principle

Antifungal activity of active principles or cell free culture filtrate was checked by radial growth bioassay, spore germination bioassay.

### 3.15.1. Spore germination

Fungal spores of test fungi were bioassayed against active principle obtained from selected bacteria on glass slides following the method of Trivedi and Sinha (1976). A drop of the test solution was placed on a clean, grease free slide and allowed to evaporate following which a drop of fungal spore suspension was placed

over it. In another slide, methanol was added in place of the antifungal compound which was treated as solvent control. The slide without any solvent but containing only spore suspension was considered as water control. The slide was incubated in a moist Petri plate for 24 h at $30^0 \pm 2^0$C. Finally one drop of lacto phenol-cotton blue was added to each spot to fix the germinated spore. The slides were observed under microscope and the percentage of germination was calculated. For each case, a total of about 200 spores were counted from about 5-8 microscopic fields.

### 3.15.2. Radial growth

The method of Fiddaman and Rossall (1994) was followed with modifications. Autoclaved PDA medium (20ml) was mixed with 0.5 ml of test compound solution and plated into petriplates (70mm dia.). After solidification, an agar block (6mm dia.) containing growing mycelium of the test fungus (4 day old) was placed in the centre of each petriplate. Control plates were mixed with solvent alone. The plates were incubated at $30^0 \pm 2^0$C and radial growth of mycelia was measured after every 2, 4, 6 and 8 days interval.

### 3.15.3. Agar cup bioassay

Antifungal activity was also determined by agar cup bioassay technique. Initially, wells were made in the periphery of the plates with a sterile cork borer and test active principle (0.2 ml) was added to each well. Following this, an agar block (2mm dia.) containing growing mycelium of the test fungal inoculums (6mm) was placed at the centre. The plate was inoculated at $30^0 \pm 2^0$C for desired period. The diameter of the fungal pathogen was noted. The plate containing 0.2 ml of dissolving solvent and plate without any solvent served as solvent control respectively. The experiment was performed in replicates of three and was repeated thrice.

### 3.15.4. Liquid medium

Cell free culture filtrate was added to the liquid medium (PDB and NB) at different percentage (20-80 %) and inoculated with test pathogen. Then whole setup was incubated at $30^0 \pm 2^0$C for 6-7 days to observe the growth of pathogen. The media without the cell free culture filtrate was treated as the control.

### 3.16. Biochemical analyses

#### 3.16.1. Chlorophyll

#### 3.16.2.1. Extraction

For the extraction of chlorophyll from leaves of plants the method of Harborne (1973) with modifications was used. 1g leaf tissues were crushed in a mortar and pestles using 80% acetone in the dark to prevent the photo oxidation of chlorophyll. The crushed samples were filtered thorough Whitman No.1 filter paper. Final volume was made up 25 ml with adding sufficient amount of acetone. A tube containing 80% acetone was used as blank.

#### 3.16.1.2. Estimation

For the estimation of chlorophyll, absorbance of the acetone extracts was measured directly at 645 nm and 663 nm in a spectrophotometer. The amount of total chlorophyll, chlorophyll a and chlorophyll b was calculated by the following formula.

Total chlorophyll = $(20.2\ A_{645}+8.02A_{663})$ μg/ml.

Chlorophyll a = $(12.7\ A_{663} - 2.69\ A_{645})$ μg/ml.

Chlorophyll b = $(22.9\ A_{645} - 4.68\ A_{663})$ μg/ml.

#### 3.16.2. Phenols

#### 3.16.2.1. Extraction

Method of Mahadevan and Sridhar (1982) was followed for the extraction of phenol. 1g of fresh leaf tissues were cut into small pieces and immediately immersed in 10 ml of 80% boiling ethanol. After 15 minutes of boiling, it was cooled and crushed in mortar and pestle followed by filtration through Whatman No1 filter paper. Final volume was adjusted with 80% ethanol. The whole extraction of phenol was done in dark to prevent induced degradation of phenol. The filtrate was used for further assay.

#### 3.16.2.2. Estimation

##### 3.16.2.2.1. Total phenol

For estimation of total phenol content of leaves, to 1 ml of the alcoholic extract, I ml of I N Folin Ciocalteau's reagent and 2 ml of 20 % sodium carbonate solution was added in test tube. The reaction mixture was shaken and heated on a boiling water bath for 1 min. After cooling under running tap water, the volume of the reaction mixture was raised to 25 ml by adding distilled water. Absorbance of the blue

colored solution was measured in a systronic photometric colorimeter model 101 at 650 nm. Quantity of total phenol was estimated using caffeic acid as standard.

**3.16.2.2.2. O – dihydroxy phenol**

For the estimation O-dihydroxy phenol content of leaves, to 1ml of alcoholic extract, 2 ml of 0.5N HCl, 1ml of Arnow's reagent ($NaNO_2$, 10g $Na_2$ $MoO_4$ – 10g distilled water – 100ml) and 2 ml of 1N NaOH was added. The reaction mixture turned pink. The volume of the reaction mixture was raised to 10 ml by adding distilled water. Absorbance of the colored solution was recorded by systronic photometric colorimeter Model 101 at 515 nm. Quantity of the O-dihydroxy phenol was estimated using caffeic acid as standard.

**3.16.3. Extraction of Enzymes.**

**3.16.3.1. β- 1, 3- glucanase** (EC .3.2.1.39)

Enzyme was extracted following method described by Pan *et al.* (1991). 1g of tea leaves were crushed in liquid nitrogen using 5 ml of chilled 0.05 M sodium acetate buffer (pH 5.0). The extracts were collected for mortar and pestle and then centrifuged at 10,000 rpm for 15min at 4°C and the supernatant was used as crude enzyme extract.

**3.16.3.2. Chitinase** (EC. 3.2.1.39)

Chitinase was extracted following the method described by Boller and Mauch (1988) with modifications. 1g of tea leaf sample was crushed in liquid nitrogen and extracted using 5 ml of chilled 0.1 M sodium citrate buffer (pH 5.0). The extract was centrifuged at 12,000 rpm for 20 min. The supernatant was collected and used as enzyme source for further assay.

**3.16.3.3. Phenylalanine ammonia lyase** (EC.4.3.1.5)

1 gm of leaf tissues was crushed in 5 ml of 0.1M sodium borate buffer (pH 8.8) containing 2 mM β– mercaptoethanol at 4°C followed by centrifugation at 12,000 rpm for 20min at 4°C. The supernatant was collected and after recording its volume, was immediately used for assay or stored at -20°C. (Chakraborty *et al.* 1993)

**3.16.3.4. Peroxidase** (EC. 1.11.1.7)

The enzyme was extract by crushing 1 gm of leaf sample in 5ml of 0.1 M sodium borate buffer (p11 8.8) containing 2mM β-meraptoethanol at 4°C followed by centrifugation at 12,000 rpm for 20min at 4°C. After centrifugation the supernatant was collected and after recording its volume was immediately used for assay or stored at -20°C (Chakraborty *et al.* 1993)

**3.16.4. Assay of enzyme activities**

**3.16.4.1. β-1, 3-glucanase**

Estimation of the β-1, 3 glucanase activities was measured 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 $40^0$ 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 preparations mixed with laminarin with zero time incubations. The enzyme activity was expressed as µg glucose liberated from laminarin by activity of enzyme from 1g tissue/min.

**3.16.4.2. Chitinase**

For estimation of chitinase activity of plants, to 10 µl of 1 M sodium acetate buffer (pH 4.0), 0.4 ml of enzyme solutions and 0.1 ml of colloidal chitin were added. Mixtures were incubated at $37^0$C for 2h then the reaction was stopped by centrifugation at 10,000 rpm for 3 min. An aliquot of supernatant was pipetted into a glass reagent tube containing 30 µl of 1M potassium phosphate buffer, pH 7.1 and incubated with 20 µl of (3% w/v) desalted snail gut enzyme Helicase (Sigma) for 1h, after which pH of the reaction mixture was brought to 8.9 by addition of 70µl of 1M sodium borate buffer (pH 9.8).The mixture was incubated in boiling water bath for 3 min and then rapidly cooled over ice. After addition of 2ml β-dimethylamino benzaldahyde (DMAB) reagent, the mixture was incubated for 20 min at $37^0$C

Immediately thereafter the absorbance value at 585 nm was measured using a UV-VIS spectrophotometer. Activity was expressed as mg N-acetyl glucosamine (GleNAc) released from chitin by activity of enzyme from 1g tissue/min.

### 3.16.4.3. Phenylalanine ammonia lyase (PAL)

PAL activity was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. For the estimation, 300µl of L-phenylalanine, 300µl of 0.0003M sodium borate (pH 8.0), 500µl of enzyme extract and 1.9ml of water was added in test tube followed by incubation at $40^0$C for 1h in water bath. The absorbance was recorded at 290nm against a bank without the enzyme in the assay mixture in UV-VIS spectrophotometer. The enzyme activity was expressed as µg cinnamic acid produced in 1 min/g fresh weight of tissue.

### 3.16.4.4. Peroxidase

For estimation of peroxidase activity in treated and untreated control plants, 100µl of freshly prepared crude enzyme extract, 1 ml of 0.2 M Sodium phosphate buffer (pH 5.4), 100 µl of ortho-dianisidine (5mg $ml^{-1}$methanol) and 1.7 ml of distilled water were added in a spectrophotometer cuvette. To initiate the reaction 0.1ml of $H_2O_2$ was added to the cuvette and changes in absorbance were recorded at 460nm in a UV-VIS spectrophotometer per minute. Peroxidase activity was expressed on fresh weight basis as changes in absorbance $min^{-1}g^{-1}$ (Chakraborty *et al.* 1993).

## 3.17. Catechins

### 3.17.1. Extraction

Catechins were extracted from tea leaf tissues following the method of Obanda *et al.* (1994) with slight modification. At first 10g leaves samples were heated in 100ml of acetone at $45^0$C in water bath for 30 min. The extracts were decanted and filtered through Whatman No. 1 filter paper. Acetone extract was concentrated to dryness and finally the residue was dissolved in 20 ml distilled water. Water solution was extracted with equal volume of chloroform for four times. The pH of the water layer was adjusted to 2 by adding two drops of 2N HCl and finally extracted with methyl isobutyl ketone. The extract was concentrated to dryness and finally dissolved in 3 ml of 2% acetic acid. The samples were filtered though milipore filter (Milipore 0.4µm HA filter paper).

### 3.17.2. HPLC analysis.

The extracted catechins were analyzed by HPLC using C-18 hypersil column with linear gradient elution system as follows: mobile phase A 100% acetonitrile, B

2% acetic acid in water. Elution 88% B for 6 min then linear gradient to 75% B over 5 min The elution was complete after a total min of 25 min. Flow rate was fixed as 1 ml $min^{-1}$ with sensitivity of 0.5 aufs. Injection volume was 20 ul and monitored at 278 nm. Peaks were compared with the peaks obtained for standard catechins. (Sigma chemicals, USA)

### 3.18. Immunological studies

### 3.18.1. Preparation of antigens

#### 3.18.1.1. Bacterial antigens

Bacterial antigen was obtained by strong sonication of bacterial cells in 0.05 M sodium phosphate buffer (pH 7.2) under chilled condition and centrifuged at 4°C for 20 minutes at 10.000 rpm. Then supernatant was collected and used as antigen for the preparation of antibody.

Prior to injection quantity of protein was measured and these was also analyzed by SDS-PAGE.

#### 3.18.1.2. Fungal antigens.

Mycelial protein was prepared following the method as outlined by Chakraborty 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 0.05M sodium phosphate buffer (pH 7.2) supplemented with 0.85% NaCl, 10mM sodium metabisulphite and 0.5mM $MgCl_2$ in ice-bath. The homogenate mixture was kept for 2h or overnight at $4^0$C and then centrifuged at 10,000 rpm for 30min. at $4^0$C to eliminate cell debris.

The supernatant was collected and stored in -$20^0$C and used as antigen for the preparation of antiserum.

#### 3.18.1.3. Soil antigens

Soil antigens were prepared following the method of Walsh *et al.* (1996). Soil samples were collected and 1gm of soil was crushed in 2ml of 0.05 M sodium carbonate–bicarbonate buffer (pH 9.6) in a mortar and pestle and kept overnight at $4^0$C. Next day centrifugation was done at 10,000 rpm for 10 min. The supernatant was collected and used as antigen for micro plate trapping and blotting purposes.

### 3.18.2. Estimation of protein content and SDS-PAGE analysis of antigenic proteins

#### 3.18.2.1. Estimation of soluble protein

Soluble proteins were estimated following the method as described by Lowry *et al.* 1951. To 1 ml of protein sample (taking $10^{-1}$ to $10^{-2}$ dilution) 5 ml of alkaline reagent (0.5ml of 1% $CuSO_4$ and 0.5ml 0f 2% sodium potassium tartarate added to 50ml of 2% $NaCO_3$ in 0.1 N NaOH) was added and incubated for 15-20 min. at room temperature. Then 0.5 ml of Folin ciocalteau reagent (diluted 1:1 with distilled water) was added and again incubated for 15 min and colour was developed following absorbance values were measured at 700 nm. Quantity of protein was measured from standard curve made with Bovine Serum Albumin (BSA)

#### 3.18.2.2. SDS-PAGE analysis of protein

Sodium dodecyl sulphate polyacrilamide gel electrophoresis was perfprmed for the detailed analysis of protein profile following the method of Laemmli (1970)

#### 3.18.2.2.1.Preparation of stock solutions

Following stock selections 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 filtered through Whatman No. 1 filter paper and was kept in brown bottle, stored at 4°C and used within one month.

**B. Sodium Dodecyle sulphate (SDS)**

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

**C. Tris buffer**

(a) 1.5 m Tris buffer i)1.5 M Tris buffer was prepared for resolving gel. (pH adjusted to 8.8 with concentrated HCl and stored at $4^0$C for use).

(b)1.0 M Tris buffer was prepared for use in the stacking and loading buffer. (pH adjusted to 6.8 with conc. HCl and stored at $4^0$C for use).

**D. Ammonium persulphate (APS)**

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

**E. E. Tris-Glycine electrophoresis buffer**

This is a running buffer and consists of 25mM Tris base, 250mM Glycine (pH-8.3) and 0.1% SDS; A 1x solution can be made by dissolving 3.02g Tris base, 18.8g glycine and 10 ml of 10% SDS in 1L of distilled water.

**F. SDS loading buffer**

This buffer consists of 5mM Tris HCl (pH 6.8), 10mM β-mercaptoethanol; 2% SDS; 0.1% bromophenol blue, 10% glycerol. A 1x solution was made by dissolving 0.5 ml of 1M Tris buffer (pH 6.8), 0.5 ml of 14.4M β-mercaptoethanol, 2ml of 10% SDS, 10mg bromophenol blue, 1ml of glycerol in 6.8ml of distilled water.

**3.18.2.2.2. Preparation of Gel**

To analyze the protein pattern through SDS-PAGE slab gels (mini) were prepared. Two glass plates (size 8cm x10cm) 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 the three sides and were sealed with high vacuum grease and clipped thoroughly to prevent any leakage of the gel solutions during pouring. Resolving gels were prepared by mixing stock solutions in the following order and poured leaving sufficient space (comb+1cm) for the stacking gel.

**10% resolving gel**: Composition of resolving gel solution of 7.5ml was as follows

| | Selections | Amount |
|---|---|---|
| a) | Distilled water | 2.95ml |
| b) | 30% Acrylamide mixture | 2.50ml |
| c) | 1.5 m Tris (pH 8.8) | 1.0ml |
| d) | 10% SDS | 0.075ml |
| e) | 10% APS | 0.075ml |
| f) | TEMED | 0.003ml |

The gel was immediately overlayered with isobutanol so that surface of gel remains even after polymerization. The solution was kept for 1h for polymerization of resolving gel. After polymerization isobutanol was poured off and washed with distilled water to remove any unpolymerized acrylamide. Then stacking gel (5%) was prepared by mixing the stock solutions.

**5% stacking gel**: Composition of gel solution was as follows:

| | Selection | Amount |
|---|---|---|
| a) | Distilled water | 2.10ml |
| b) | 30% Acrylamide mixture | 0.50ml |
| c) | 1.0 m Tris (pH 6.8) | 0.38ml |
| d) | 10% SDS | 0.03ml |
| e) | 10% APS | 0.03ml |
| f) | TEMED | 0.003ml |

Stacking gel solution was poured over the resolving gel and comb was inserted immediately leaving a space of 1 cm between resolving gel and comb and overlayered with water. The gel kept for 30 min. After polymerization of stacking gel the comb was removed and washed thoroughly. The gel was then mounted in the electrophoresis apparatus after removing the spacer at the bottom. Tris-glycine running buffer was added sufficiently in both upper and lower reservoir. Precaution was taken to remove any bubble trapped at the bottom of gel.

**3.18.2.2.3. Sample preparation**

Sample was prepared by mixing the sample protein (34µl) with 1 x SDS gel-loading buffer (16µl) in cyclomixer. All the samples were floated in boiling water bath for 30mim to denature the protein sample. The samples were immediately loaded in a predetermined order into the bottom of the well with T-100 micropipette syringe. Along with the samples, protein marker consisting of a mixture of six proteins ranging in molecular weight from high to low (phosphorylase-b-97, 400; bovine serum albumin- 58,000; ovalbumin-43, 000; carbonic anhydrase- 29,000; soybean trypsin inhibitor-20, 000; lysozyme-14, 300 daltons) was treated as the other samples and loaded in separate well.

**3.18.2.2.4. Electrophoresis**

Electrophoresis was performed at constant 18mA current for a period about 3h until the dye front reached the lower end of gel.

**3.18.2.2.5. Fixing and Staining**

After completion of 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 250mg of coommassie brilliant blue (Sigma R250) in 45ml methanol. When the stain was completely dissolved 45ml of distilled 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 fixer and stained in this staining solution for 4h at 370C with constant shaking at very low speed. After staining, the gel was finally destained in destaining solution containing methanol, distilled water and acetic acid (4.5: 4.5:1) at 400C with constant shaking until background became clear.

**3.18.3. Raising of polyclonal antibodies**

**3.18.3.1. Rabbits and their maintenance**

Polyclonal antibodies were prepared against fungal and bacterial antigens in New Zealand white male rabbits approx. 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 Animal House (Antisera Reserve for Plant Pathogens), IP Lab., Dept. of Botany, NBU. They were regularly fed with green grass, soaked gram, carrot and green vegetables like cabbage, carrots etc, and twice a day. Beside this, they were given saline water after each bleeding for three consecutive days. Cages and floor were cleaned everyday in the morning for better hygienic conditions.

**3.18.3.2. Immunization**

Method of Alba and Devay (1985) and Chakraborty and Saha (1994) were followed for immunization. Before immunization, normal sera were collected from rabbit. For developing antisera, intramuscular injections of 1 ml antigen emulsified in equal volume of Freund's complete adjuvant (Genei) were given into each rabbit 7

days after pre-immunization bleeding and repeating the doses at 7days intervals for consecutive week followed by Freund's incomplete adjuvant (Genei) at 7days intervals upto 12-14 consecutive weeks as required.

### 3.18.3.3. Bleeding

The rabbits were bled by marginal ear vein puncture. First bleeding was taken 3 days after first six injections and then subsequently seven times more every fortnight. During bleeding the rabbit was placed in their backs on a wooden board, fixed at $60^{O}$ angles. The neck of the rabbit was held tightly in the triangular gap at the edge of the board and the body was fixed in such a way that the rabbit could not move during the bleeding. The hairs were removed from upper side of the ear followed by disinfection with spirit. Then the ear vein was irritated by the application of xylene and incision was made with sharp sterile blade and 5-10 ml of blood sample was collected in sterile graduated glass tube. After collection, all precautionary measures were taken to stop the flow of the blood from the puncture. The blood sample was kept at $37^{O}$C for 1h for clotting. Then the clot was loosened with a sterile needle and antiserum was taken and clarified by centrifugation at 5,000 rpm for 10 min at room temperature. Finally, antisera were distributed in 1 ml vial and stored at $-20^{O}$C. These antisera were used for Double diffusion analysis, Dot blot analysis and Enzyme Linked Immunosorbent Assay (ELISA).

### 3.18.4. Purification of IgG

### 3.18.4.1. Precipitation

IgG was purified by affinity chromatography on a DEAE cellulose column following the method of 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 $22^{O}$C for h. Then the precipitate was dissolved in 2 ml of 0.02 M sodium phosphate buffer, pH 8.0.

### 3.18.4.2. Column preparation

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

**3.18.4.3. Fraction collection**

At the top of the column, 2ml of ammonium sulphate precipitate was applied and the elution was performed at a constant pH and a molarity continuously changing from 0.02 M to 0.03 M. The initial elution buffer (1) was 0.02 M sodium phosphate buffer (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 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 molarity. Ultimately, 40 fractions each of 5 ml were collected and the optical density values were recorded at 280 nm using UV-Vis spectrophotometer (DIGISPEDC 200GL).

**3.18.5. Immunodiffusion.**

**3.18.5.1. Preparation of agarose slides**

Glass slides (6 cm x 6 cm) were degreased in 90% v/v ethanol; ethanol: diethyl ether (1:1 v/v) and ether then dried in hot air oven. After drying the plates were sterilized inside the petridish each containing one plate. Agarose gel was prepared in 0.05 M Tris–barbiturate buffer (pH 8.6). The buffer was heated within a conical flask placed in a boiling water bath 0.9% agarose was mixed to the hot buffer and boiled for the next 15 min. The flask was repeatedly shaken thoroughly in order to prepare clean molten agarose and 0.1 % (w/v) sodium azide was added into it. For the preparation of agarose gel, the molten agarose was poured on the sterilized glass slides (10 ml slide$^{4}$) in laminar flow chamber and kept 15 min for solidification. After the 3-7 wells are cut out with a sterilized cork borer ( 6 mm diameter) at a distance of 1.5 cm – 2 cm away form central well and 2.0 – 2.5 cm from well to well ( peripheral).

**3.18.5.2. Diffusion**

Agar gel double diffusion tests were carried out using antigen and anfiserum following the method of Ouchterlony (1976). The antigen and undiluted antisera ( 50 µl/well) was pipette directly into the appropriate well in a laminar chamber. The

diffusion was allowed to continue in a moist chamber for 72 h at 25°C. Precipitation reaction was observed in the agar gel only in cases where common antigen was present.

**3.18.5.2.1. 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 0.1% $NaN_3$) for 72 h with 6 hourly changes to remove unreacted antigens and antisera widely dispersed in the agarose gel. Then slides were stained with Coomassic brilliant blue (R 250, Sigma; 0.25 g Coomassic blue, 45 ml methanol, 45 ml distilled water and 10 ml 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 ratio) with changes until the background became clear. Finally slides were washed with distilled water and dried in hot air oven for 3 h at 50°C.

**3.19. Determination of bacterial sustainability in soil**

The sustainability of bacteria in the rhizosphere soil was determined by immunological techniques.

**3.19.1. Enzyme Linked Immunosorbent assay (ELISA).**

ELISA tests as out lined by Chakraborty *et al.* (1995) were carried out using following buffers.

1. Antigen coating buffer-Carbonate-Bicarbonate buffer 0.05M pH 9.6

**Stocks**

A. Sodium carbonate-5.2995g in1000ml dist. water.

B. Sodium bicarbonate-4.2g in 1ooml dist. water.

160ml of stock A was mixed with 360ml of stock B and pH was adjusted to 9.6.

2. Phosphate buffer saline: 0.15M PBS pH-7.2

**Stocks**

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

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

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

3. 0.15M phosphate buffer saline-Tween (0.15M PBS-Tween, pH7.2)

To 0.15M PBS, 0.05% Tween-20 was added.

4. Blocking reagent (Tris buffer saline, pH 8.0)

   0.05 M Tris, 0.135M NaCl, 0.0027 M KCl

   Tris -0.657g

   NaCl-0.81g

   KCl -0.223g

Distilled water was added to make up the volume to 100ml. Then pH was adjusted to 8.0 and 0.05% Tween 20 and 1% Bovine serum albumin (BSA) were added.

5. Antisera dilution buffer (0.15M PBS-Tween, pH 7.2)

In 0.15 M PBS- Tween, pH 7.2, 0.2%BSA, 0.02% Polyvinylphyrrolidone, 10,000(PVPP 10,000) and 0.03% sodium azide ($NaN_3$) was added.

6. Substrate.

P- Nitrophenyl phosphate (Himedia) 1mg/ml dissolved in substrate buffer (1.0% w/v, diethanolamine, 3mM $NaN_3$, pH 9.8).

7. 3 N NaOH solution was used to stop the reaction.

**3.19.1.1. Direct antigen coated (DAC) ELISA.**

DAC-ELISA was performed following the method as described by Chakraborty *et al.* 1995. Antigens were diluted with coating buffer and the antigens were loaded (200 µl per well) in ELISA plate (Coaster EIA/RIA. Strip plate, USA) arranged in 12 rows in a (Cassette) ELISA plate. After loading, the plate was incubated at 25°C for 4 h. Then the plate was washed 4 times under running tap water and twice with PBS- Tween and each time shaken to dry. Subsequently, 200 µl blocking reagent was added to each well for blocking the unbound sites and the plate was incubated at 25°C for I h. After incubation, the plate was washed as mentioned earlier, Purified IgG was diluted in antisera dilution buffer and loaded (200 µl per well) to each well and incubated at 4°C overnight. After a further washing, goat antirabbit IgG labeled with alkaline phospatase (Sigma chemicals, USA, in 1: 10,000 dilution with PBS), was added to each well (200 µl per well) and incubated at 37°C for 2 h. The plate was washed dried and loaded with 200µl of p-Nitrophenyl

Phosphate substrate in each well and kept in dark for 45-60min.Color development was stopped by adding 50µl per well of 3N NaOH solution and the absorbance was determined in an ELISA Reader (Multiskan, ThermoLabsystems) at 405 nm. Absorbance values in wells not coated with antigens were considered as blanks.

**3.19.2. Dot Blot**

Dot Blot was performed following the method suggested by Lange et al (1989) with modification. Following buffers were used for dot blot;

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

(ii) Tris buffer saline (10mMpH 7.4) with 0.09% NaCI and 0.5 % Tween-20fo washing.

III Blocking solution 10 %( w/v) skim milk powder (casein hydrolysate. SLR) in TBST 0.05 Tris – HCL, 0.5

IV Alkaline phosphatase buffer (100 mM Tris HCI. 100 mM NaCI, 5mM $MgCI_2$

**Assay**

Nitrocellulose membrane (Millipore, 7cmx10cm, Lot No.: H5SMO 5255, pore size 0.45um Millipore corporation, Bedford) was first cut carefully into the required size and placed inside the template. 4µl of coating buffer was loaded into each well of the template over the NCM and was kept for 30 min. to dry. Following this 4µl of test samples (antigen) was loaded into the template wells over the NCM and was kept for 1 h at room temperature. Template was removed and blocking of NCM was done with 10% non fat dry milk (casein hydrolysate, SRL) prepared in TBST for 30 – 60 minutes on a shaker. Respective polygonal antibody (IgG 1:500) prepared against that antigen was added directly in the blocking solution and further incubated at $4^{O}C$ for overnight. The membrane was then washed gently with running tap for 3 min. followed three time 5 minutes washes in TBST (pH 7.4) (wakeham and white, 1996). The membrane was then incubated in alkaline phosphatase conjugated goat antirabbit IgG (diluted 1: 10000 in alkaline phosphatase) for 5 h at $37^{O}C$. The membrane was washed as before, 10 ml of the NBT/BCIP substrate (Genei) was added next and color development was stopped by washing the NCM with distilled water and color development was categorized on the intensity of dots.

### 3.20. Determination of pathogen in soil by immunological methods

For detecting the presence of pathogen in the soil, the protein samples from pathogen inoculated soil, pathogen and *B. pumilus* inoculated soil as well as uninoculated sterile soil was extracted. For extraction of protein 5 g of soil was taken in 10 ml of protein extraction buffer [0.05 M sodium phosphate buffer (pH7.2)]. The soil was stirred for 24 h followed by crushing and centrifugation as previously mentioned. The protein samples collected from different soil samples were used for immunological detection as mentioned below.

#### 3.20.1. ELISA

The protein extracted from pathogen inoculated soil samples was analyzed by ELISA technique as mentioned before.

#### 3.20.2. Dot-blot

The determination of presence of pathogen in soil inoculated with pathogen as well as *B. pumilus* and pathogen inoculated soil was done by Dot-blot technique using antigen prepared from the soil as mentioned above.

### 3.21. Preparation of tale – based PGPR formulation and its application in the soil

For development of bio-formulation 10 g of CMC was mixed with I kg of talcum powder and pH was adjusted to 7.0 by adding calcium carbonate. It was then sterilized for 30 min in two consecutive days. To this sterilized talcum powder 400 ml of bacterial inocula containing $3x10^9$cfu/ml was added and mixed well under sterile condition. The talc mix was dried under shade to bring moisture to less than 20%. The formulation was packed in milky white color polythene bags, sealed and stored at room temperature for future use.

The talcum based formulation was applied in the field at the rate of 100 g per pot. The experiment was conducted to assess the effect on shoot growth, increase in number of leaves etc. due to the application of tale-based formulation.

Bioformulation was also made in the tea waste. In case of tea waste bioformulation, the waste was soaked in water overnight to remove excess phenols. This was then dried and procedure as above was adopted.