Chapter III

Materials and Methods
The present study was carried out under the agro-climatic condition of Jorhat, Assam, India, at the experimental nursery of Biotechnology Division, Regional Research Laboratory (CSIR) during the year 2002 to 2005. The experimental site was situated between 26°47’N latitude and 94°12’E longitude at an elevation of 86.60 m above mean sea level. Hot and humid summer, dry and cold winter is common features of this region.

The methodology followed and the materials used in the present study are detailed below.

**Soil:**

The experiments were conducted on sandy-loam soil with pH 4.63, total nitrogen of 0.015 per cent, organic carbon 0.268 per cent and having no previous history of any pesticide or synthetic agrochemical application.

**Plant:**

Three year old tea (*Camellia sinensis* (L.) O. Kuntze) cuttings (Clone: TV 1) obtained from Tata Tea R&D Centre, Teok, Jorhat, were used for the entire study.

**Organisms selected:**

**Bacterial isolates:**

**RRLJ 134:** The *Pseudomonas fluorescens* strain RRLJ 134 was obtained from the culture collection of Biotechnology Division of RRL, Jorhat, originally isolated from the top soil (pH 5, sandy loam) of a tea plantation in the Dooars region (26°44’ N & 88°55’ E), West Bengal, India. The final confirmation of the identification of this strain was done from MTCC Division of Institute of Microbial Technology (CSIR), Chandigarh, India and the Accession number of this strain is 7522.
RRLJ 04: *Pseudomonas aeruginosa* strain RRLJ 04 was isolated from the rhizoplane of paddy (*Oryza sativa* L.) from Bhavnagar, Gujarat, India. The MTCC Accession number of this strain is 7277.

**Fungal isolates:**

The fungal pathogens *Fomes lamoensis* (culture no. 4140) and *Ustulina zonata* (culture no. 4144) were procured from Indian Type Culture Collections (ITCC) of Indian Agricultural Research Institute (IARI), New Delhi, India.

**Objective no. 1: Selection of potential strains for induction of systemic resistance against brown root and charcoal stump rot diseases along with biomass improvement of tea under nursery condition.**

1.1 *In vitro* antibiosis test with live organism:

*In vitro* antagonism test was done by two methods: Line inoculation and Spot inoculation.

1.1.1 Line inoculation:

This was done according to Dileep Kumar (1998). King’s medium B (KB), Nutrient Agar (NA) and Potato Dextrose Agar (PDA), were used for examining the antagonism. For this, an actively growing mycelial disc (approx. 6mm²) was placed at one side of the Petri plate, 2 cm inside the periphery and a loopful the rhizobacterial strain was streaked in a line on the opposite side at a distance of 5 cm from the mycelial disc. The plates were incubated at 28±2°C and inhibition zone was measured as distance (in cm) between the respective rhizobacterial test antagonist and *F. lamoensis*, *U. zonata* fungal pathogen after 7, 16 days of growth respectively.
1.1.2 Spot inoculation:

For this, an actively growing mycelial disc (approx. 6mm²) was placed in the centre of the Petri plate and a loopful of the rhizobacterial strain was spot-inoculated on the periphery of the Petri plate, 2.0 cm inside at two opposite equidistant places and incubated at 28±2°C. Inhibition zones were measured as distance (in cm) between the respective rhizobacterial test antagonist and fungal pathogen *F. lamoensis, U. zonata* fungal pathogen after 7, 16 days of growth respectively.

**Treatments:**

All the laboratory as well as nursery experiments were done with the following treatments throughout the entire period of investigation:


**Objective no. 2: Bacterization of tea cuttings for development of induced systemic resistance against brown root and charcoal stump rot diseases beside biomass improvement in gnotobiotic and nursery condition.**

2.1 Growth promotion and disease suppression studies under gnotobiotic condition:

This was done according to Barthakur and Bezbaruah (1999). The system consisted of an indigenously fabricated chamber consisting of a glass tube of 32 cm length and 5 cm in diameter with a 15 cm narrow tube of diameter 2 cm connected to it at the bottom. This was inserted in a 500 ml conical flask (Fig. 2).
Fig. 2. Glass system for gnotobiotic studies

The tube was filled with a mixture of soil and sand (3:1) up to 22.5 cm by securing it with a piece of muslin cloth at the bottom of the tube. This was then fixed tightly to the conical flask containing 450 ml of Hoffland's plant nutrient solution (PNS)*. The bottom portion of the tube was immersed in the PNS to about 6.5 cm to keep the sand column moist. The system was autoclaved at 121°C for 20 min. After autoclaving, the cooled system were introduced with surface sterilized (with 1% HgCl₂) tea cuttings under a laminar flow. The experiment was set up with the treatments as specified above. In control only PNS was given so as to moist the soil. In rest of the treatment, respective bacterial (24 h old) and/or fungal broth culture (F. lamoensis, 10 days old; U. zonata 20 days old) @10 ml / tea cutting was given and the soil was saturated with the PNS. From
time to time PNS was added in the flask to wet the soil throughout the experimental period. The results were recorded up to 10 days of growth.

*PNS: 5mM Ca(NO₃)₂; 5mM KNO₃; 2mM MgSO₄·7H₂O; 1mM KH₂PO₄; 100 ml micronutrient solution per 1 litre PNS.

Micronutrient solution (g/l): MnSO₄ – 0.6; ZnSO₄ – 0.50; H₃BO₃ – 1.27; Na₂MoO₄ – 0.40; CuSO₄ – 0.20

Since the tea cuttings did not survive for more than 10 days in the above mentioned system even in control, the glass chamber was replaced with a conical flask. Here, also the experiment was performed under aseptic condition with the same set of treatments as mentioned above. The tea cuttings were placed in autoclaved conical flask containing 450 ml of PNS amended with bacterial and/or fungal broth culture @ 50 ml / flask as per treatment specification. The data were recorded up to 15 days.

2.2 Growth promotion and disease suppression studies under nursery conditions:

2.2.1 Seed bacterization:

Seed bacterization was done according to Dileep Kumar and Bezbaruah (1996). For this, bacteria grown on KB medium for 48 h were scraped with a sterile glass rod and mixed with moist sterile soil to prepare a paste (approximately 1.0 x 10⁷ cells/g soil). The cuttings were placed in this paste for 12 h and 5 g of the soil paste was attached around the root portion of the cutting before planting. Cuttings treated with moist soil without any bacteria served as control. Both treated and control cuttings were planted in polythene bags containing field soil and maintained under nursery conditions.

The experiment was laid out in Completely Randomized Design (CRD) as indicated earlier with three set of replication. Under each replication 25 numbers of tea cuttings were treated as per treatment. In case of treatment RRLJ 134 and RRLJ 04, the tea
cuttings were bacterized and planted in the polythene bags (21 X 15 cm) filled with a mixture of soil and Farm Yard Manure (FYM) in 3:1 ratio. Tea cuttings of the treatment *F. lamoensis*, *F. lamoensis* + RRLJ 134, *F. lamoensis* + RRLJ 04, *U. zonata*, *U. zonata* + RRLJ 134 and *U. zonata* + RRLJ 04 were challenge inoculated with the homogenized broth culture of respective pathogens, *F. lamoensis* and *U. zonata* (10, 20 days old respectively) @50 ml/cutting. Cuttings treated with sterilized water served as the control. Proper hygienic condition under the nursery was maintained by weeding, light forking and irrigation as and when required to maintain the healthy condition of tea cuttings and to minimize the interference of external factors other than desired. After a month of planting, tea cuttings were again treated twice with respective pathogen and PGPR strains as per different treatment specification at 15 days interval. At 60 days after treatment five numbers of representative tea cuttings were selected randomly and data on growth promotion in terms of increase in shoot height, numbers of leaves were taken before uprooting of tea cuttings. These cuttings were then uprooted from the polythene bags with utmost care to keep the roots intact and washed gently under running tap water to remove the adhering soil particles. Data on root length, chlorophyll contents of leaves, fresh and dry weight of shoot, root and leaf & a bud were recorded. Occurrence of disease symptom was observed by recording the wilting of leaf leading to drying. Disease incidence was recorded upto 90 days of treatment. Per cent Disease Incidence (PDI) and Per cent Disease Control (PDC) were calculated out based on disease incidence.

### 2.2.2 Extraction and estimation of chlorophyll content in leaves:

The extraction and estimation of chlorophyll content (mg/g) of leaves was done according to Mahadevan and Sridhar (1986).
Objective no. 3: Isolation of promising bioactive metabolites and their utilization in growth promotion and/ induction of disease resistance in tea under gnotobiotic condition.

3.1 Isolation of bioactive metabolites:

The bioactive compounds were isolated according to Dileep Kumar and Bezbaruah (1997). A bacterial lawn of PGPR strain was grown in KB media for 7 days at 28±2°C. The bacterial grown medium (greenish blue) was then cut into small pieces (≈1cm²) and extracted in 80% aqueous acetone till the medium turned into colourless. The solvent was passed through 2 layers of cheese cloth and then the acetone was removed. The resultant aqueous part was then treated with sodium chloride (50 g/L) and centrifuged (12,000g for 20 min). The supernatant was collected and extracted with diethyl ether (3:1 v/v) in a separating funnel. The aqueous fraction was then treated with chloroform (3:1 v/v) and the chloroform fraction was collected by discarding the aqueous portion. The diethyl ether and chloroform fractions were evaporated and the dried extract was then used for further studies. Since bioactive metabolites produced by RRIJ 134 and RRIJ 04 were found similar in nature with same effect on the test pathogen in vitro condition, further studies were done with only two number of bioactive metabolites-diethyl ether fraction and chloroform fraction, irrespective of the source organism.

3.2 In vitro antibiosis test with bioactive metabolites:

For this sterilized filter paper were impregnated with different concentrations of bioactive metabolite (0, 10, 20 mg bioactive metabolite /100 ml methanol) and in vitro antagonism was examined as in case of spot inoculation with live organism. Sterilized filter paper disc impregnated with methanol served as control.
3.3 Effect of bioactive metabolites under gnotobiotic condition:

The two fractions so obtained were studied for their effect on induction of systemic disease resistance in tea cuttings under gnotobiotic condition. For this, each bioactive metabolite was separately dissolved in 2 ml sterile distilled water to give a concentration of 20 % were taken as stock solutions. The same procedure as described in live organism was followed here with the bioactive metabolites. Initially to standardize the amount of metabolite required, different concentrations (25, 50, 75 and 100 µl per 100 ml PNS) were applied to the tea cuttings. The concentration in which the cutting was able to survive more than the only PNS (control) was taken as the optimum concentration (each for diethyl ether and chloroform fraction) and were further applied for detailed studies.

For induction of systemic resistance with bioactive metabolites, the following treatments were given under gnotobiotic condition:


The cuttings were observed for occurrence of disease symptoms upto 15 days.

Objective no. 4: Study the mechanism(s) of crop enhancement and/or induction of systemic resistance in host plant.

To study the mechanism of development of ISR the following experiments were done.

4.1 Split root experiment:

4.1.1 Under gnotobiotic condition:

Following treatments were given under this experiment:

Under each treatment, two numbers of autoclaved culture tubes of length 19.5 cm, diameter 3.0 cm was taken and held together with the help of a rubber band (Fig. 3).

![Fig. 3. Set up of split root experiment under laboratory condition](image)

After this the experimental set up was placed under aseptic *in vitro* condition and two different treatments were given in each tube, to see the effect of one treatment on the other without direct contact. The tubes were first filled with 90 ml PNS and volume was made upto 100 ml with the respective PGPR strain or the pathogen as per treatment. Tea cuttings having distinct bifurcation of root were selected and surface sterilized. Tea
cuttings were put in the combined culture tubes in such a way that some part of the surface sterilized root enters one tube and other part in the other tube. Care was taken to dip the root portions sufficiently in the treatments given.

In case of treatment *F. lamoensis : F. lamoensis* and *U. zonata : U. zonata*, a well homogenized 10 and 20 days old broth culture of *F. lamoensis* and *U. zonata* respectively, were given in both the culture tubes. In treatment RRLJ 134 : RRLJ 134 and RRLJ 04 : RRLJ 04, respective broth culture of PGPR strains (24 h old) were given in both the tubes. In case of treatment *F. lamoensis : RRLJ 134, F. lamoensis : RRLJ 04, U. zonata : RRLJ 134 and U. zonata : RRLJ 04* respective pathogen and PGPR strains were put separately in two adjacent culture tubes. Both the tubes with PNS served as control. Data on leaf drying, leaf shedding and drying of tea cuttings were recorded upto 12 days of treatment. Since the tea cuttings didn't survive beyond 12 days so the same experiment was repeated under nursery condition with the following modifications:

4.1.2 Under nursery condition:

Here the culture tubes were replaced with the polythene bags of size 21 x 15 cm and in them two separate chambers were made by placing transparency sheet in between (Plate 1). The polythene bags were filled with a mixture of sterilized soil and FYM in 3:1 ratio in the two separate chambers. Tea cuttings with distinct separate roots were surface sterilized and put in the polythene bags in such a way that one portion of the root lie in one section and other portion in the adjacent section of soil separated by the transparency sheet. The treatments as mentioned above, were given after the planting of cuttings.
Plate 1. Set up of split root experiment under nursery condition (with transparency sheet)

In case of treatment *F. lamoensis* : *F. lamoensis* and *U. zonata* : *U. zonata*, a well homogenized 10 and 20 days old broth culture of *F. lamoensis* and *U. zonata* respectively were applied @ 10 ml in both the separated soil sections. In treatment RRLJ 134 : RRLJ 134 and RRLJ 04 : RRLJ 04, respective broth culture of PGPR strains (24 h old) @ 10 ml were applied in both the separated sections of the same polythene bag. In case of treatment *F. lamoensis* : RRLJ 134, *F. lamoensis* : RRLJ 04, *U. zonata* : RRLJ 134 and *U. zonata* : RRLJ 04, the respective pathogen and PGPR strains were put separately in two adjacent soil sections. Both the soil sections treated with PNS served as control. Proper care was taken to ensure that soil particles of one portion do not mix with the other. Data on leaf drying, leaf shedding and drying of tea cuttings were recorded up to 50 days of treatment.

In this experimental set up, though the two soil sections were separated with a piece of transparency and utmost care was taken not to mix the soil particles of the two chambers, but the migration of pathogen and/or PGPR strain from one section of soil to
other couldn’t be ruled out. To avoid this, the experimental set up was further modified as follows:

The polythene bag of 21 x 15 cm size were sealed in middle with the help of an electrical sealer so as to get two equal separate halves (Plate 2). Rest of the experimental set up and treatments applied were same as described under gnotobiotic condition. Same set of data as already described in the above experiment was recorded upto 60 days of treatment.

Plate 2. Set up of split root experiment under nursery condition (with sealed packet)

4.2 Enzyme level studies:

For the better understanding of mechanism of induced systemic resistance, enzyme level studies were done. Pathogenesis related two enzymes viz., β,1-3-glucanase and Polymethyl galacturonase and defense related enzymes viz., L-phenylalanine ammonia lyase (PAL), Peroxidase (POX) and Polyphenol Oxidase (PPO) were studied as follows:
4.2.1 β,1-3-glucanase (EC 3.2.1.4):

4.2.1.1 Preparation of enzyme extract:

This experiment was laid in two sets, in first set, three numbers of conical flasks each containing 100 ml of potato dextrose broth (PDB) amended with 2% carboxymethyl cellulose (CMC) and adjusted to pH 6.5, was inoculated with *F. lamoensis*. In the second set, another three numbers of conical flask were inoculated with *U. zonata*. Under each set, two numbers of flasks, already inoculated with respective fungal culture, were inoculated with RRLJ 134 and RRLJ 04 respectively after 8 and 16 days of *F. lamoensis* and *U. zonata* growth respectively. After 10 days of incubation, cultures were then filtered through buchner funnel using Whatman No.1 filter paper and centrifuged at 2000 g for 40 min. A few drops of toluene was added to the filtrate and used as the enzyme source.

4.2.1.2 Enzyme assay:

Enzyme assay in terms of per cent loss in viscosity was done according to the method of Mahadevan and Sridhar (1986). 4 ml of CMC (0.5 gm of CMC in 100 ml of sodium acetate-acetic acid buffer at pH 5.2) and 2 ml of the enzyme was pipette into viscometer kept in a water bath at 30°C. The contents were mixed by drawing air gently through the large arm of the viscometer. Suction was applied to the small arm and efflux time of the mixture was determined at 10 min interval. The per cent loss in viscosity of CMC under different treatment was calculated according to the following formula:

\[
V = \frac{T_0 - T}{T_0 - T_{H2O}} \times 100
\]

where, \(V\) = per cent loss in viscosity, \(T_0\) = flow time in seconds at zero time, \(T\) = flow time of the reaction mixture at time \(T\), and \(T_{H2O}\) = flow time of distilled water.
Based on the per cent loss in viscosity of CMC under each treatment, per cent reduction in viscosity loss caused by respective pathogen in presence of respective PGPR strains were calculated as follows:

\[
\text{% viscosity loss by pathogen - % viscosity loss in presence of PGPR} \times 100 \\
\text{ % viscosity loss by pathogen}
\]

4.2.2 Polymethyl galacturonase (EC 3.1.1.11):

4.2.2.1 Preparation of enzyme extract:
For preparation of pectic enzyme extract, same procedure was followed as mentioned above in case of cellulase enzyme except the use of the 4% pectin in place of CMC.

4.2.2.2 Enzyme assay:
This was done according to Mahadevan and Sridhar (1986). 4ml of 1% pectin solution (1g of pectin in 100 ml acetate buffer and pH 5.2, adjusted with 1N HCl or 1N NaOH) was pipetted in a viscometer. One ml of acetate buffer and 2 ml of the enzyme source were added to it and gently mixed by drawing air rapidly through the large arm of the viscometer. Suction was applied through the small arm and the efflux time of the mixture was determined. This was the zero time. The efflux time of the mixture was measured at an interval of 10 min. The per cent loss and per cent reduction in viscosity of pectin under different treatment was calculated using the formula mentioned in cellulase assay.

To study the change in the level of defense related enzyme viz., PAL, POX and PPO in the host plant under different treatments with progress of time after treatment, the experiment was laid out in CRD with 9 treatments and 3 numbers of replications as mentioned in earlier nursery experiment. Under each treatment 25 numbers of tea cuttings were treated. Under each treatment as per treatment specification 50 ml of well
homogenized 10 and 20 days old broth culture of pathogen (*F. lamoensis* and *U. zonata* respectively) and/or 24 h old PGPR strain (RRLJ 134 or RRLJ 04) was applied per cutting. Enzyme assay was done at 10 days interval upto 50 days after treatment. A sample was taken by detaching healthy leaves from each treatment and extraction and assay of different enzymes (PAL, POX and PPO) were done as described below:

### 4.2.3 L-phenylalanine ammonialyase (PAL) (EC 4.3.1.5):

#### 4.2.3.1 Preparation of enzyme extract:

Leaf samples were collected and kept in ice-box. 3 g of leaf material was then ground in a pre-cooled (4°C) mortar and pestle containing 9 ml of sodium borate buffer mixed with 2- mercaptoethanol (0.8 ml/lit. of buffer). The extract was centrifuged at 12,000 g for 20 min. The supernatant was used as enzyme extract for the enzyme assay. The samples were stored on ice after centrifugation till enzyme assay.

#### 4.2.3.2 Enzyme assay:

PAL activity was determined as described below using the method of Sadasivam and Manickam (1991). Assay of PAL was done within 3 h of grinding the samples, during which time no loss in activity was detected. 0.5 ml sodium borate buffer, 0.2 ml of enzyme extract and 3 ml distilled water were taken in a test tube. To this, 1 ml L-phenylalanine solution was added to initiate the reaction. The tube was incubated for 45 min at 32°C. Then 0.5 ml of 1M trichloroacetic acid was added to stop the reaction. For control, phenylalanine was added after trichloroacetic acid. The absorbance was measured at 290 nm. The reaction rate was expressed as micromole trans-cinnamic acid formed per 100 g fresh weight per min, which was calculated from the standard curve of trans-cinnamic acid of known strength.
4.2.4 Peroxidase (POX) (EC 1.11.1.7):

4.2.4.1 Preparation of enzyme extract:

One gram of fresh leaf was ground in a pre-cooled mortar and pestle containing 3 ml of 0.1 M phosphate buffer (pH 7.0). The extract was centrifuged at 18,000 g at 5°C for 15 min. The supernatant was used as enzyme extract for the enzyme assay. The samples were stored on ice after centrifugation till enzyme assay.

4.2.4.2 Enzyme assay:

POX activity was determined using a modified method of Thimmaiah (1999) as described below. Assay of POX was done by incubating 1 ml O-dianisidine, 0.5 ml of \( \text{H}_2\text{O}_2 \), 1 ml of phosphate buffer and 2.4 ml of distilled water in a test tube at 30°C. For blank (control), \( \text{H}_2\text{O}_2 \) was excluded but additional volume of water was added. The reaction was started by adding 0.2 ml of the enzyme extract and stopped after five min by the addition of 1 ml 2N \( \text{H}_2\text{SO}_4 \). Absorbance was recorded at 430 nm. Total activity of peroxidase enzyme was expressed as units/min./100g fresh weight of sample considering one unit of enzyme as an increase in OD by 1.0 under standard conditions.

4.2.5 Polyphenol oxidase (PPO) (EC 1.14.18.1):

4.2.5.1 Preparation of enzyme extract:

One gram of fresh leaf was ground in a pre-cooled mortar and pestle containing 2 ml of 0.1 M sodium phosphate buffer (pH 7.1). The extract was strained through 4 layers of cheese cloth and the filtrate was centrifuged at 15000 g at 6°C for 20 min. The supernatant was collected and used as enzyme extract for the assay. The samples were stored on ice after centrifugation till enzyme assay.
4.2.5.2 Enzyme assay:
Polyphenol oxidase (PPO) activity was determined according to the method of Sadasivam and Manickam (1991). For this, 2.5 ml of 0.1 M phosphate buffer (pH 6.5) and 0.3 ml of catechol solution (0.01 M) were taken. The spectrophotometer was set at 495 nm. To the above mixture, 0.2 ml of enzyme extract was added and the change in absorbance was recorded at 30°C for every 30 sec up to 5 min. The activity of PPO was expressed as the change in absorbance per ml of enzyme extract per min at 495 nm under the assay conditions. PPO activity in units per g fresh weight was then calculated and estimation was done in triplicate.

4.3 Production of antibiotics:
Bioactive metabolites from respective PGPR strains were isolated according to Dileep Kumar and Bezbaruah (1997). The bioactive metabolites were further purified by thin layer chromatography and various analytical studies were done.

4.4 Production of siderophore:
For detecting siderophore production, the test fluorescent pseudomonads were first grown in KB broth for 18 h to get a culture which was used to inoculate succinate medium*. 500 ml flasks containing 200 ml succinate medium were inoculated with the test organism grown on KB broth and incubated on a rotary shaker (120 rpm) for 48 h. The cultures were centrifuged at 10,000 g for 20 min and the supernatant obtained was examined for its absorption spectrum between 200-600 nm on a UV-visible spectrophotometer. A peak at or near 405 nm indicated the presence of siderophores.

*Succinate medium: Succinic acid – 4.0; (NH₄)₂SO₄ – 1.0; KH₂PO₄ – 3.0; K₂HPO₄ – 0.1; MgSO₄.7H₂O – 0.2; pH – 7.0
Objective no. 5: Large scale nursery and field experiments with successful PGPR strains to induce systemic resistance and biomass improvement in tea plants.

5.1 Large scale nursery experiment:

The experiment was conducted in the same manner as done in case of earlier nursery condition but instead of 25 numbers of tea cuttings, 100 numbers were taken in each replication. Occurrence of disease incidence was recorded under each treatment at an interval of 15 days. Data on different growth promotion parameters were recorded at 90 days after first treatment from randomly selected 10 numbers of tea cuttings.

5.2 Field experiment:

Brown root rot disease could not be found under field condition after extensive field survey since it is a primary root disease and in tea gardens secondary root disease such as charcoal stump rot was found restricted in small pockets. On investigation, it was found that plants were mainly attacked by charcoal stump rot disease as the plants become weak due to primary root disease caused by *F. lamoensis* or by lightning strikes. To see the effect of a bioformulation based on these strains on charcoal stump rot disease under field condition, an experiment was laid out with three replications.

5.3 Preparation and spray of bioformulation:

For preparation of this formulation, the organisms were grown separately in a medium containing Urea 6.0, Molasses 20.0, KH$_2$PO$_4$ 3.0, MgSO$_4$.7H$_2$O 1.0 (g/L) for seven days. After the growth period the bacterial cells were killed by acidification (5% HCL) of the broth and mixed together. Collar region of tea bushes were drenched with (250 ml/bush) this bioformulation.
After that 60 liter of this formulation diluted to 600 liters with water and sprayed to bushes in one hectare at an interval of one month for two times and data on the disease incidence was recorded at weekly interval for the next one year.

Objective no. 6: Root colonization study through drug resistant mutants.

6.1 Selection of antibiotic resistant strains:
To monitor the presence or colonization of roots by the rhizobacteria, their antibiotic-resistant strains were developed according to Dileep Kumar (1998). For this, the strains were streaked on KB medium amended with a mixture of streptomycin sulphate and chloramphenicol in the ratio of 50:50 mg/l. The strains that grew on the amended medium and showed the characters of parental types were selected for further studies.

6.2 Root colonization by the introduced bacteria:
This was done according to Dileep Kumar (1998). Tea cuttings bacterized with drug-resistant strains of RRLJ 134 and RRLJ 04 (designated as RRLJ 134+ and RRLJ 04+ respectively) was sampled for root colonization by the introduced bacteria upto 49 days at an interval of 7 days.

For this, one g of root (cut into approximately one cm segments) from bacterized plants was dipped in 5 ml sterile distilled water and shaken for 3-4 min to release the rhizoplane bacteria into the water. Appropriate dilutions of the bacterial suspensions were made and pour plated on (i) KB medium amended with streptomycin sulphate and chloramphenicol (50 mg/l each) for enumerating the introduced bacteria, and (ii) NA for the total aerobic bacteria on the rhizoplane. The colony forming units (CFU) for one g of fresh root segments were calculated after 48 h of incubation.
6.3 Population dynamics of RRLJ 134\(^+\) and RRLJ 04\(^+\) on the tea roots:

The population of the introduced bacteria that colonized on the inoculated tea cutting roots was studied for 60 days at an interval of 10 days. For this, fresh roots of tea cuttings bacterized with RRLJ 134\(^+\) and RRLJ 04\(^+\), respectively, were cut into (0.5cm size) different segments starting from the origin towards the tip, and grouped separately. From each lot, one g of root was dipped in 9 ml sterile distilled water and shaken vigorously for 3-4 min to get the colonized bacteria in suspension. After serial dilution, from \(10^{-5}\) dilution, 1ml suspension was pour plated on KB medium containing streptomycin sulphate and chloramphenicol (50 mg/l each) and the population of the introduced bacteria at different distances from the root origin (0-2, 2-4, 4-6, 6-8 and 8-10 cm) were calculated in terms of CFU/g of fresh root.

6.4 Microscopic observation of \textit{F. lamoensis} and \textit{U. zonata} on tea roots:

Tea cuttings treated with \textit{F. lamoensis} and \textit{U. zonata} respectively under nursery condition and showing disease symptoms were carefully uprooted. Roots were washed in running tap water and thin transverse sections (T.S.) were made. These sections were observed under microscope for detection of presence of fungal mycelium associated with root section. Diseased root sections were surface sterilized in 90\% alcohol and placed in potato dextrose agar media amended with streptomycin sulphate (@100mg/l) for isolation of the fungus. Fungus isolated was again observed under microscope for identification and the confirmation was done from Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi.

6.5 Microscopic observation of \textit{F. lamoensis} and \textit{U. zonata} in PDA media:

For this study, PDA media was amended with 2\% cotton blue. A thin layer of this amended media was poured in sterilized slides. Mycelium of respective pathogen
isolated from diseased root sections grown in PDA slants was mixed thoroughly in 10 ml of sterile distilled water. From this, one loopful was inoculated in the amended PDA media and covered with a cover slip and observed under the microscope periodically to see the gradual growth pattern of the respective pathogen.

**Objective no. 7: Purification and characterization of promising bioactive metabolites.**

Diethyl ether and chloroform fractions isolated as described earlier were further purified by thin layer chromatography (TLC) as follows:

**Diethyl ether fraction:** A mixture of toluene and ethyl acetate in 4:1 ratio was selected as the solvent system to run the preparative TLC plates. The separated fractions were cut and eluted with methanol. The solvent were removed under reduced pressure and the pure compounds were dried in vacuum.

**Chloroform fraction:** The compounds in this fraction were separated in two phases. Initially one compound was isolated by running the extract over preparative TLC plates using ethyl acetate as the mobile phase. The remaining isolated base part was separated again by ethyl acetate and methanol (9:1) as the mobile phase. The TLC plates were dried under vacuum. Various analytical methods *viz.* IR, NMR and mass spectroscopy were done for the identification of the isolated compounds.

**Statistical analyses**

All the data obtained were subjected to various statistical methods as given by Gomez & Gomez (1984) and Panse & Sukhtame, (1989).