Chapter 3
METHODOLOGY

3.1 Identification of Study area:

Random survey was conducted among agricultural field and forests in various locations in sub Himalayan area of Jalpaiguri District ($26^{o}15'47''$ & $26^{o}59'34''$ N Latitude and $88^{o}23'2''$ & $89^{o}7'30''$E Longitude) of North Bengal in the State of West Bengal. The average rainfall is from 2500mm-3000mm*, 80% of which falls during monsoon and about 110 rain days. Soil is acidic to neutral in nature and low in fertility. The criteria of location selection were based on prior study of areas where target crops (pulses and beans) are being cultivated. (* source IMD)

3.2 Soil sampling and collection

The soil sample was collected from different locations, after considering a minimal depth of 20cm to 25cm using sterile spatula. Initial soil layer was removed manually by hand hoe. The samples were then transferred under sterile condition in Ziplock plastic bags and stored in airtight plastic containers (Cello) with ice packs in polypropylene ice box at (4^{0}C to 5^{0}C) and transported to Laboratory (2 hrs).The samples were then pre-treated and stored in incubator for future usage.

3.3 Determination of pH

The soil sample from various locations was tested to determine the pH of the soil. pH is important for the occurrence and inhabitation by respective microflora. The determination was done by help of digital pH meter.(Systronics)

3.4 Sterilization & cleaning of Glassware

The glassware was cleaned with chromic acid solution by soaking them in the solution overnight. Then 3% commercially available Lysol solution was used to treat the glassware. Finally washing was done by running tap water repeatedly. After drying the glassware were kept in a hot air oven for 3-6 hours at 140-180°C. Sterilizations of the glassware were done by autoclaving them.

3.5 Isolation of Microorganism from soil rhizosphere

The soil samples were pre-treated to facilitate isolation of actinomycetes and avoid contamination by other microorganism. The samples about 100 gm in each slot were air dried, heated aseptically in laminar air flow by layering the soil sample to 2cm and incubated in chamber at 28^0C for two days (Gebreyohannes *et al.*,2013). The treated samples were crushed in already baked mortar,sieved and stored for immediate use.

3.5.1 Soil dilution technique

Warcup's soil dilution method (1955) was followed for isolating microorganisms from the rhizosphere with certain standarized modifications. The soil suspension was prepared by dissolving 10g of sample in 30 ml sterile saline water and blending with a magnetic stirrer for 1hr. The suspension was allowed to settle, until distinct layers were visible, one of the sediment and other of the supernatant.

Then, following method of Masayuki *et al*., (1988), with some modification, Stock solution was prepared by diluting 1gm of sediment in 9ml of sterile saline water and shaken again in vortex mixer. 1 ml was taken to prepare the final volume of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} by serial dilution method.

3.6. Screening of Actinomycetes from rhizosphere soil

0.1 ml of suspension from each dilution of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} was taken with the help of sterile micro pipette and were evenly spread on previously prepared Starch casein nitrate agar media and Oatmeal Agar (ISP Medium 3) under aseptic conditions. Replicates, three in number, were used for plating each sample. The setting was incubated at 28^0C for 7 to 21 days (Masand *et al*.,2015), under constant observation and as per growth of the microorganism under sterile conditions.

Media composition

SCN Media (Starch- Casein-Nitrate)

Soluble Starch.....10gm
Casein.....0.3gm
Potassium nitrate.....2gm
Sodium chloride.....2gm
Di Potassium hydrogen phosphate.....2gm
Magnesium sulphate.....0.05gm
Calcium carbonate.....0.02gm
Agar.....15gm
Dist.water.....1000ml
P^H 7

Oatmeal Agar (ISP Medium 3)

Oatmeal.....20gm
Agar.....18gm
Trace salt soln.....1.0gm
Dist water.....1000ml

Trace salt solution

$Feso_4$.....0.1gm
$MnCl_2$.....0.1gm
$ZnSO_4$.....0.1gm
Dist. H_2O.....100ml

Water and oatmeal was boiled and simmered for 20 minute. The solution was filtered through cheese cloth and agar was added and volume was supplemented back to 999ml, brought to boil and autoclaved at 121^0C for 15 minute, and 1ml trace salt solution was added aseptically.

3.7 Maintenance and preservation of isolates

Pure colonies were screened visually, identified, and isolated under sterile conditions and was maintained in Starch casein nitrate agar slants at 4^0C for future studies.

3.8 Assessment of actinomycelial growth

17 isolates from pure colonies were obtained after screening 72 plates from different locations. The maintained slants were used to streak and stab isolates in both liquid and solid media:

3.8.1. Solid media: Isolates were streaked in Starch- Casein-Nitrate Agar media and was incubated at 28^0C for seven days with constant observation.

3.8.2. Liquid media: Isolates were streaked in Starch- Casein-Nitrate broth in Erlenmeyer flask and was incubated at 28^0C for seven days with constant observation, after shaking in rotary shaker for 30 minutes.

3.9 Classical Approach for characterization of isolates

Both macroscopic and microscopic methods as outlined within the identification key of Guide to the Classification and Identification of the Actinomycetes and Their Antibiotics (Selman and Hubert 1953) and Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons,1974) were followed for characterization of isolates.

3.9.1 Morphological method

To study the isolates, strains were transferred from 7 day old mother culture and was inoculated into 1.5% agar medium as stabs and incubated at 37^0C. Upon observation that the isolate had suspended and mixed in media (semi solid), 2 drops from medium was pipetted onto a sterile glass slide in laminar air flow. Subsequently, 1 to 3 drops of agar was spread well on the slide. When a thin film was formed, the setting was incubated at 27^0C under sterile conditions. Observation was made under microscope at timely interval.

3.9.1.1. Spore chain morphology (Microscopic morphology)

Spore chain was characterized morphologically after grouping into 'sections'. (Sivakumar *et.al*, 2005) The species belonging to genus *Streptomyces* was divided into three sections (Shirling and Gottlieb,1966), those being rectifexibiles (RF), retinaculiaperti (RA) and Spirales (S). When two types of chains were present for the same strain, both types were considered.

3.9.1.2.Study of Aerial mycelium and substrate mycelium (Macroscopic morphology)

The colour of the aerial mycelium bearing spore was observed by the naked eye and the colour of the spore mass was noted .If the spore mass have two different colour mixed up then both the colour were noted. For these isolates were grown in Oatmeal Agar media (ISP 3) for 7 days at 28ºC and the colour of the isolates were recorded.

Reverse side pigments: Isolates were streaked on Oatmeal agar media and incubated at 28ºC for 7 days. Pigmentation on the reverse side of the colony was noted as distinctive (+) and non distinctive (-) and the isolates were divided in these two groups.

3.9.1.3. Study of Diffusible pigments

For the study of diffusible pigment production the isolates were grown in Liquid media for a duration of 7 days. The isolates were divided into two groups on the basis of their ability to produce soluble pigment.

3.9.1.4. SEM studies of isolates

For scanning electron microscopy, pellet collection was done after centrifugation (3000 rpm) of isolate culture grown in nutrient broth medium and simultaneous washing in 0.1M phosphate buffer saline. Prefixing of the sample, under vacuum, was carried in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 6.8). Passing through different alcohol grades with initiation from the lower grade was done for dehydrolysis of the samples. Critical point drying in CO_2 (CPD 030; BAL TEC Vaduz, Liechtenstein), was undertaken after mounting the sample on slab and coating with 20 nm silver palladium alloy. JEOL JSM 5200 Scanning Electron Microscope (Tokyo, Japan) was used for the examination.

3.10. Biochemical characterization of isolates

3.10.1. Gram reaction

Gram staining was done by the method as described by Buchanan and Gibbson(1974) with some modifications. Actinomycetes were grown in SCN plates. After incubation period of 3 days one sterile coverslip was placed on the actinomycetes colony aseptically. The impression on the coverslip was air-dried and heat-fixed. Then the coverslip was flooded with crystal violate solution(crystal violate 2.0gm, 95%alcohol 20ml, ammonium oxalate 1%W/V, aqueous solution-80ml) and kept for 1 minute. Then the coverslip was flooded with Burke's iodine solution (iodine 1.0gm, KI 2.0gm, distilled water 100ml) and kept for 1minute. Then slides were washed with distilled water for 5seconds and 95% alcohol was poured drop by drop keeping the coverslip in 45º angle until the excess stain comes out. Again washed with distilled water and finally counter stained with safranine (2.5 W/V safranine in 95% alcohol- 10ml, distilled water 100ml.) for 2 minutes washed with water and dried. The gram reaction and morphological characters were observed under oil immersion objective.

3.10.2. Starch degradation

The bacteria were streaked on sterilized starch agar plate (NA + 0.1% soluble starch) and incubated for five days at 37º C. The plates were flooded with Lugol's iodine solution. The clear zone underneath and around the growth indicates the starch hydrolysis.

3.10.3. Catalase production

24 hour old bacterial culture was flooded with 0.5 ml 10% H_2O_2 solution and formation of gas bubbles indicated the positive reaction.

3.10.4. Indole production.

Inoculation of isolate was done in 10ml of Davis Mingoli's broth which was supplemented by 0.1% tryptophan. Incubation was done at 37^0C for duration of 7 days. Careful layering of the culture was done by 2 ml of Ehrlich bobme reagent (P- dimethlyaminobenzaldehyde 10g in 100 ml concentrated HCL.) on the surface. This was allowed to stand for a few minutes and was observed. The formation of a ring at the interface of medium and reagent if any indicated the production of Indole.

3.10.5. H_2 S production

Streaking of isolates was done on SIM agar slants and incubation undertaken for 48hrs at an optimum of 37^0C. Visible darkening (affect) along the inner lines of the slant walls was taken as indicative positive result for H_2S production.

3.10.6. Urea digestion

Colour change of the medium was taken as indicator for the presence of urease. Streaking of isolates about half a loop unto medium was done and the set with paraflim cover was incubated for a duration of 7 days at controlled condition of 37^0C.

3.10.7. HCN production

Procedure described by Wei *et al.,* (1991) with standardized modifications was followed for determination of hydrocyanic acid (HCN) production.

During the follow up of procedure, NA medium with glycine (4.4g L^{-1}) amendment in petri plate was used to grow bacteria. Whatmann filter paper was cut in thin strips which were in turn soaked in picric acid solution. The papers were attached on to the lid of the plates and was sealed with parafilm. The setup of the same in replicates was incubated for 2 to 4 days. Change in colour of the filter paper strip, from brown to red was taken as indicator for ability of the isolate to produce HCN.

3.11. Physiological characterization of isolates

3.11.1. Growth of isolates at different salt concentration

Growth of the isolates at different NaCl concentration was determined by growing the isolates in SCN media supplemented with 1%, 2%,5%,8% and 10% (w/v) NaCl. Isolates were streaked onto petriplates having SCN media supplemented with 1%, 5% and 10% (w/v) NaCl and incubated at 28ºC for 5days. (Sahin *et al*.,2002)

3.11.2. Resistance of isolates to different antibiotics: Screening of the isolates towards resistance to different antibiotics was performed by Agar diffusion method as described by Sahin *et al.* (2002) with some modifications. Antibiotic discs were placed on the inoculated SCN plates. Plates were incubated for 5 days at 28ºC and the zone of growth inhibition was measured.

3.12. *In vitro* Screening and evaluation of phosphate solubilizing activity of isolated microorganism.

3.12.1. Qualitative analysis for phosphate solubilizing activity (screening)

Actinomycetes isolates were screened in Pikovskaya medium for phosphate solubilization activity (Pikovskaya,1948). Isolates were streaked in Pikovskaya media and incubated at room temperature for 7 days. A clear halo zone around the growth indicates phosphate solubilization activity. The plates were observed for clear zone around the colony and diameter of the halo zone was measured.

3.12.2.Quantitative analysis for phosphate solubilizing activity (Evaluation)

Phosphate solubilising activity of respective isolates and its evaluation was undertaken by culturing the isolates in Pikovskaya's liquid medium, which in turn was supplemented by 0.5% tri Calcium phosphate and 0.5% rock phosphate separately.The flasks were constantly shaken in rotary incubator at 100rpm and kept for 10 days at 28^0C.Kundsen and Beegle's (1988)Amoniummolybdate ascorbic acid method was followed for estimation of phosphate. Amount of the phosphate utilized or solubilized by the isolates were expressed as mg/L phosphate utilized by deducting the amount of residual total phosphate from the initial amount of phosphate source added to the modified Pikovskaya liquid medium (yeast extract- 0.5 gm/L, dextrose- 10.0gm/L, Calcium phosphate/ Rock phosphate- 5.0gm/L, ammonium phosphate 0.5gm /L, Potasium chloride- 0.2gm/L, Magnesium sulphate- 0.10gm/L, Manganese sulphate - 0.0001gm/L, ferrous sulphate- 0.0001gm/L, pH- 6.6). 7days old culture grown on SCN

slants was taken as 5%v/v of actinobacterial suspension and inoculated in 50/250 ml (v/v) broth. The broth upon centrifugation at 10000 rpm was allowed to settle and the supernatant was collected. Mixing of 2 ml of aliquot and 8ml colorimetric solution (60gm/L Ammonium paramolibdate, 1.455gm Antimony potassium tartarate, 700ml/L conc.H_2SO_4 and 132ml/L of ascorbic acid) was done in a thorough basis and incubated for 20 min till colour development took place. Percent transmittance of the solution was taken on a colorimeter with wavelength set at 882nm.

3.13. IAA production.

3.13.1. Quantitative test for IAA production

To enhance acetic acid (IAA) production Tryptophane (0.1mM) was used in a high C/N ratio medium, wherein isolate were grown for 24 to 48 hrs (Prinsen *et al.*, 1993).Assay in culture supernatant was done for IAA by Pillet- Chollet method described by (Dobbelaere *et al*., 1999). Reagent preparation and composition was done by adding 12g $FeCl_3$ per litre in 7.9 M H_2S0_4. 1ml of the recently pepared reagent was added to 1ml sample supernatant and was mixed well, kept in the dark for 30 minutes at room temperature. Absorbance was measured at 530nm.

3.14. *In vitro* Screening and evaluation for antibacterial activity

Cross streak method was applied to assess antibacterial activity. Single streak at one end of the on SCN plate was done. Incubation was done for 7 days and seeding allowed by a single streak in perpendicular to the plane of the previous one. Due observance of size of inhibition zone upon interaction of microbial strains, upon further incubation was done and recorded (Lertcanawanichakul and Sawangnop, 2008).

3.15. *In vitro* Screening and evaluation for antifungal activity

3.15.1. Inhibition of fungal mycelial growth in solid media

The efficacy of individual isolates was tested *in vitro* for inhibiting growth of the pathogen in dual culture using PDA and SCN media. Each actinomycetes isolate was placed at one side of the agar plate about 1cm away from the edge and 7mm diameter block of the pathogen taken from growing edge of the fungal culture was inoculated at the other half of the Petri plate. In another experiment actinomycetes were placed circling the pathogen. For each test three replicate plates were used. The plates were incubated for 7 days (depending upon the growth of the pathogen) at 28ºC and inhibition zone towards the fungus colony in individual plate was quantified.

Results were expressed as mean % of inhibition in presence of the actinomycetes isolate (Gamliel *et al*.1989) The percent inhibition in the radial colony growth was calculated by the following formula:

$$\% \text{ inhibition} = 100 - (R^2 \times 100 / r^2)$$

Where r= Radius of growth of pathogen in control: R=Radius of growth of pathogen in treated set

3.15.2. Inhibition of fungal growth by metabolites

Extraction of antifungal compounds from Actinomycetes

Extraction of the metabolites from the actinomycetes grown in solid media was carried out with some modification of the method described by Soares *et al*., (2006). The actinomycetes were grown in solid argentine glycerol agar (AGS) medium at 28°C for 7 days. After that 10 ml sterile distilled water was added aseptically to each petriplate containing the actinomycetes and the plates were incubated for 24hours at room temperatute. The metabolite suspension obtained was centrifuged at 12000rpm for 15 min. After centrifugation the supernatant was collected and cold sterilized by passing through sterilized micro filter (0.22 µm pore size). The metabolites suspensions were transferred to sterile glass vials and kept at 4°C until further use.

3.15.2.1. Effect on Fungal mycelial growth

The method of Soares (2006) was followed with modification. Autoclaved PDA medium (20ml) was mixed with 0.5ml of test compound solution and plated into petriplates (70 mm dia.). After solidification, 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 30ºC ± 1 ºC and radial growth of mycelium was measured after every 2, 4, 6 and 8 days interval.

3.15.2.2. Effect on fungal spore germination

Fungal spores of test fungi were bio assayed against Secondary metabolitesobtained from selected isolates on glass slides following the method of Soares *et al.,* (2006). 40µl of the metabolites suspension was taken in a grooved glass slide to which 40 µl of fungal pore suspension was added. The control treatment was prepared with sterile distilled water instead of metabolites suspension. The slide was incubated in a moist Petri plate for 24 h at 30ºC ± 1 ºC. Finally one drop of lacto phenol - cotton blue was

added to each spot to fix the germination of spore or conidia. The slides were observed under microscope and the percentage of germination was determined.

3.15.2.3. Effect on Sclerotia germination

For assessing the effect of the cell free culture filtrate on the sclerotial germination of *S. rolfsii* the sclerotia were scrapped off from the culture growing in 7 days old PDA plates. The sclerotia were then soaked 1 hour in cell free culture filtrate solution. The sclerotia soaked in sterile distilled water and in uninoculated sterile PDB served as the control. After soaking the sclerotia were transferred asceptically to the petri plate containing sterile Black paper. These sterile black papers were also soaked in culture filtrate for at least 30minutes and incubated at room temperature. Percent germination as well as the radial growth of the germinating sclerotia was measured.

3.16.Qualitative test for Chitinase production

Conventional plate method in chitinase detection agar (CDA) was followed to determine the secretion of chitinase. Composition included 1% (w/v) colloidal chitin with 15g of agar in medium (Na_2HPO_4 -6.0g, KH_2PO_4 -3.0g, NaCl -0.5g, NH_4Cl 1.0g, Yeast extract- 0.05g and distilled water 1L; pH 6.5). Incubation of the inoculated organism was done on CDA plate at 28^0C for duration of 7 to 10 days. Evidently the occurrence of clear zone around the growing zone is indicative of chitinase activity (Kamil *et al.,* 2007). The colloidal chitin was prepared by following the method described by Mathivanan *et. al.,*(1997). Slow addition of chitin powder (5gm) was done to 60 ml of concentrated Hydrochloric acid at 4^0C ; kept under vigorous stirring overnight. The mixture was added to 2L of ice cold 95% ethanol with rapid stirring and kept overnight at 25 ºC. The precipitation formed was collected by centrifugation at 7000rpm for 20 minutes at 4 ºC and washed with sterile distilled water until the colloidal solution become neutral (pH 7). The prepared colloidal solution (5%) was stored at 4 ºC until further use.

3.17.Isolation of genomic DNA

Isolation of actinomycetes genomic DNA was done by growing the isolates in Nutrient broth(NB) for 3-4 days. Liquid nitrogen was used for crushing the cell mass. Isolation of genomic DNA was done following the method of Muthu et al.,(2013) with some modifications.

3.17.1. Preparation of genomic DNA extraction buffer.

At desired pH the following buffers for DNA extraction was prepared from appropriate composition of desired chemicals.

Lysis Buffer
50 mM Tris, pH 8.0
100 mM EDTA
100mM NaCl
1% SDS
Genomic DNA Buffer
10 mMTris, pH 8.0
0.1 mM EDTA

3.17.2. Extraction of actinomycetes genomic DNA

The target isolates were grown in nutrient broth for 3-4 days and other isolation procedures were followed. This involved initial centrifugation of culture in broth for 5 minutes at 28^0C at 10000 rpm. The pellet in the tube after separation was collected for future use and supernatant discarded. The pellets so collected was washed carefully with distilled water about three to four times and then re-suspended in 0.5 ml of lysis solution of 100mMTris-Hcl (pH 7.5), 20 mM EDTA, 250mM NaCl and 2% SDS and 1mg/ml lysozyme.To it 5µl of RNAse(50mg/ml) was added and incubated at 37ºC for 3 hours.10µl of Proteinase K solution (20mg/ml) was added to it and further incubated at 65ºC for 3 min. Extraction of the lysate was done with equal volume of tris water saturated phenol:chloroform:isoamyl alchohol (25:24:1) and centrifugation carried out at 10,000 rpm for 5 minutes, the resulting aqueous phase was then transferred into a fresh tube and DNA precipitation was done with double volume of chilled ethanol (100%). Then DNA was pelleted by centrifuging at 10000 rpm for 5 min at 4^oC and subsequent air drying was carried out, the resulting DNA was dissolved in 40 µl of TE buffer and stored at 4^oC.

3.17.3. Purification of genomic DNA

Total genomic DNA extraction of the isolated microorganism as per the above procedure was followed by RNAase treatment. In doing so resuspension of the genomic DNA was done in 100 µl1 X TE buffer and incubated at at 37^oC for 30 min with RNAse (60µg). After due incubation, re-extraction of the sample was done with PCL solution (Phenol: Chloroform: Isoamylalcohol 25:24:1), and RNA free DNA was precipitated with chilled ethanol as was done and described earlier.

3.17.4. Spectrophotometric quantification of genomic DNA

Measurement of DNA Concentration using Spectrophotometry

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

1 O.D. at 260 nm for double-stranded DNA = 50 ng/ul of dsDNA

1 O.D. at 260 nm for single-stranded DNA = 20-33 ng/ul of ssDNA

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

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

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

3.17.5. Agarose gel eletrophoresis to check DNA quality.

Gel electrophoresis is an important molecular biology tool which allows us to study DNA. At the same time it is instrumental in determining the sequence of nitrogen bases, size of any insertion or deletion, finding presence of a point mutation if any, and also has usage in distinguishing variable sized alleles at a single locus as well as assesses the quality and quantity of DNA present in a sample.

3.17.6. Preparation of DNA samples for electrophoresis

Preparation of agarose gel for DNA gel electrophoresis, involved melting a specific amount (0.8%) of agarose in 1X TBE buffer, cooling the solution and casting the same in gel tray with ethidium bromide and solidifying of the Gel for 15-20 minutes.

3.17.7. Run gel electrophoresis for DNA fraction

Electric supply was applied at a constant current of 90mA and voltage 75 volt (BioRAD Power Pac 3000) by firmly attaching the electrical lead of the gel tank, for 90 minutes.

The DNA migrated from cathode to anode and run was continued until the bromophenol blue had migrated an appropriate distance through the gel. Then electric current was turned off and gel was removed from the tank and examined on UV transilluminator and photographed for analysis.

3.18. Sequencing of r DNA

The sequencing was done using r DNA. Bi-directional mode using ITS primer pairs by CROMAS was followed for DNA sequencing, resulting in chromatogram generation. This provided detailed and relevant information about the sequence.

Sequence analysis

Bio-informatic algorithms tool was used for analysis of DNA sequence information was using MEGA 4.

Chromatogram of sequence

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

Editing and alignment of sequence data

Editing of the DNA sequences was performed using Bio Edit software and alignment was done by using ClustralW algorithms.

3.19. BLAST of Sequence

DNA sequence analysis based on different characteristic for microorganism identification was carried out using alignment software of BLAST algorithm (http://ingene2.upm.edu.my/Blast, Altschul *et al.*, 1997). The homology of sequence was used as basis for identification of microorganism. (http://ncbi.nlm.nih.gov/blast).

3.20. Submission of rDNA Sequence to NCBI GenBank

The DNA sequences were deposited to NCBI GenBank through BankIt procedure, and approved as per the ITS sequence, after complete annotation and provision of collection of accession numbers.

3.21. Multiple sequence alignment and Phylogenetic analysis

The sequenced PCR product was aligned with extype isolates' sequences from NCBI GenBank for identification as well as for studying phylogenetic relationship. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal., 1973).

The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and these are represented in the units of number of base substitutions per site. Codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted in MEGA-4 (Tamura *et al*., 2007). 16S rDNA of all the three isolates were aligned to study the range of homology present in the conserved regions following the ClustalW algorithm (Thompson *et al.,* 1994) using the Bioinformatic tool BioEdit.

3.22. Plant Material

Seed samples of *Vigna radiate* and *Phaseolus vulgaris* were collected from local markets, and other sources. Preliminary seed germination test, viability test under new agro-climatic zone at Immune Phyto-Pathology laboratory in North Bengal University were conducted at random . One seed variety of *Vigna radiata,*and two seed variety of *Phaseolus vulgaris* was selected for proper identification and further experimental set up.Set up was made for seed propagation and plant growth both in field and pot at open and glass house condition.

Plant materials

***Vigna radiata*:** Cultivar 1 (CV1):Mung/ Paheli Dal (common name)

***Phaseolus vulgaris*:** Cultivar 2 (CV2):Rajmash/ Jwala (common name)

Cultivar 3 (CV3): Rajmash/Kholar (common name)

Plant materials were maintained in the open field (Front Line Demonstration) premises of Immuno-Phytopathology laboratory, Department of Botany, N.B.U. and Glass house for pot experiments for selected field treatment of inoculants.

3.23. Selection of potent isolates

On the basis of their different attributes like PSA, IAA production, biocontrol activity potent isolates will be selected for *in vivo* study

3.24. *In Vivo* study of efficiency of selected isolates on plant growth promotion

3.24.1. Application of actinomycetes

3.24.1.1. Seed coating

For seed coating or seed bacterization the isolates were grown in NB for 48 h at 28ºC and centrifuged at 12,000rpm for 15 minute. The cell pellet was suspended in sterile

distilled water. The optical density of the suspension was adjusted using UV-VIS spectrophotometer to obtain a final density of 3 $X10^6$cfu ml^{-1}. Surface sterilized seeds of *Phaseolus vulgaris* and *Vigna radiata* dried under sterile air steam were soaked in the cell suspension using 0.2% sterilized carboxymethylcellulose as an adhesive. Seeds were soaked overnight and next day seeds were sown following method of Errakhi *et al.*,(2007) with some modification.

3.24.1.2. Soil drench

For soil drench the method of Karimi *et al*.,(2012) was followed. The isolates were grown in NB for 48 h at 28ºC and centrifuged at 12,000rpm for 15 minute. The cell pellet obtained, was suspended in sterile distilled water. The optical density of the suspension was adjusted using UV-VIS spectrophotometer to obtain a final density of 3 $X10^6$cfu ml^{-1} .

The cell suspension was applied to the plants in both pots and field. Soil was autoclaved prior to sowing of seeds. Applications were made by distributing 100 ml per pot at regular interval of 15 days. In the field the rhizosphere of plants were flooded with the suspension.

3.24.1.3. Foliar spray

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

Growth promotions in seedlings were evaluated after 15day in terms of increase in height, shoot and root length, number of leaf, leaf area of the treated as well as untreated control plants under the same environmental and physical conditions (temperature 20±5ºC, RH 60-80%)

As both the plants belong to the Family Fabaceae they have the ability to produce root nodule to symbiotically fix atmospheric nitrogen with help of nodule forming bacteria *Rhizobium* sp. The effect of isolates on formation of root nodule expressed in terms of Nodulation index was also observed according to the formula suggested by Ben Rebah *et al.*,(2002).

Nodulation index=A x B x C ≤ 18

Nodule size = A (small=1, medium=2, large=3)

Nodule colour = B (white=1, pink=2)

Nodule number= C (few=1, several=2, many=3)

3.24.2. Pathogen inoculum preparation and application

3.24.2.1. Preparation of inoculum

The inoculum of pathogen *Fusarium solani* and *Sclerotium rolfsii* was prepared in sand maize meal media which increases the survival capacity and viability of the inoculum in soil, and inoculation of healthy plants was done. The following preparations were used for experimental purposes:

Sand maize meal medium

Marked quantity of washed and sterilized sand: water: maize meal ratio of (9: 1.5: 1, w:w:v) was taken in either autoclavable plastic bag or conical flasks (150g), and was sterilized at 20lb for 20 minutes for future use upon inoculation and mass culture of fungal pathogen (Biswas and Sen, 2000).Care was taken to select mycelia bits from margins and transition zones of actively growing culture, and the whole mass was incubated at 28ºC$\pm$2º C for 15-20 days. Inoculum was found viable for pathogenicity when it was two weeks olds under optimum condition, soil application involved 100gm inocula at the rhizosphere of each of the potted plants. Proper irrigation and aeration was maintained for optimal condition for pathogen growth.

3.24.2.2. Disease assessment

Determination of the effects of selected bacterial isolates on disease reduction was done by categorisation of the various treatment under the following types: i. Untreated control, ii. Inoculated with pathogen, iii. Inoculation with actinomycetes isolates, iv.Inoculation with both actinomycetes and fungal pathogen.

The rhizosphere of plants pre- treated with the antagonists or without treatment was inoculated with pathogen. In pre- treated plants, pathogen inoculation was done 3 days after application of antagonist. Disease assessment was performed after 7, 14, 21, 28 days of inoculation.

Above ground and underground symptoms was used for assessment of the disease intensity and its recording (Roots, colour, rotting, leaves withering, shoot tip withering, defoliation etc.) Disease intensity was calculated by using 0-6 scale as adopted by Mathew and Gupta (1996). The disease infections observation were recorded in a continuous 0-6 scale, where

0 = no symptoms:

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

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

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

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

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

6 = Whole plants die, since 100% roots were

Open Field condition

Table 5.The arrangement (dummy) of the plants in Field condition.

Vigna radiata/ Phaseolus vulgaris	Control	Isolate treatment		
Healthy (H)	UH	T_1H	T2H	T3H
Inoculated (I)	UI	T_1I	T2I	T3I

T1= Treated with *Streptomyces griseus*

T2= Treated with *Streptomyces tricolor*

T3= Treated with *Streptomyces flavogriseus*

Six blocks in replicates of three each was made.

Disease Incidence (DI%)

For calculating the Disease Incidence, the disease index was recorded based on the score 0-6, depending on above ground and underground symptoms. Disease Incidence was calculated as per methodology described by Xue *et al*., (2013) using the following formula:

Disease Incidence (DI%)=[∑(the number of plants on this index x Disease index)/(Total number of plant investigated x highest disease index)] x 100%

3.25. Extraction and assay of defence enzyme activity in treated plants.

3.25.1. Associated changes in Phenylalanine ammonia lyase (PAL) activity extraction and estimation

Extraction of PAL (EC.4.3.1.5) was done by following the method described by Chakraborty *et al.,* (1993) with modifications. Using 0.1M sodium borate buffer pH 8.8 (5ml/gm) with 2mM of ß mercaptoethanol under ice cold conditions in a mortar and pastel 1gm sample was crushed, traces of sea sand was used to make available a fine slurry, which in turn was centrifuged in 15000 rpm for 20 minutes at 4^{o}C. The supernatant so collected after due recording of its volume was used for assay else stored at 20^{0}C.

Assay of Phenylalanine ammonia lyase activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The reaction mixture contained 0.3 ml of 300µM sodium borate (pH 8.8), 0.3 ml of 30 µM L-phenylalanine and 0.5ml of supernatant along with 1.9 ml distilled water making a total volume of 3ml. Following incubation for 1 h at 40 ºC the absorbance at 290nm was taken against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as µg cinnamic acid produced in 1 min g^{-1} fresh weight of tissue.

3.25.2. Associated changes in β-1,3 glucanase activity, extraction and estimation

Extraction of ß -1,3 glucanase (EC.3.2.1.39) was done following the method described by Pan *et al.* (1991). Crushing was done in liquid nitrogen using 1g of plant leaf sample at 4 ºC using mortar and pastel, using 5ml of chilled 0,05 M sodium acetate buffer (pH 5.0) as the extraction buffer. Centrifugation was then carried out at 10000 rpm for 15 minutes at 4 ºC then supernatant was collected and used as crude enzyme extract. Estimation of the ß-1,3- glucanase was done by following the Laminarin-di-nitro-salicylate method (Pan *et. al.,* 1991). Equal volume of crude enzyme extract at 62.5µl was added to 62.5 µl of laminarin (4%) and incubation was done at 40ºC for 10 minutes and reaction was allowed to take place, which was stopped by adding 375µl dinitrosalicylic reagent and simultaneous heating was carried out for a duration of about 5 minutes over conventional boiling water bath.

The treated solution mixture was in turn diluted with 4.5 ml of water, vortexed and absorbance was recorded at 500nm. The blank taken was a mixture of laminarin and

crude enzyme with zero time incubation. The enzyme activity was expressed as µg glucose released per min per gm fresh tissues.

3.25.3. Associated changes in Chitinase activity.

Extrection of chitinase (EC. 3.2.39) was done by following the method described by Boller and Mauch (1988) with modifications. Leaf sample of 1gm weight from identified plants was crushed in liquid nitrogen, using 5ml of chilled 0.1M Sodium Citrate (pH5) as extraction buffer. The resulting homogenate was centrifuged for 10 min at 12,000rpm and the supernatant so collected was used as enzyme source.

Chitinase activity was measured according to the method described by (Boller and Mauch,1988). 10µl Na-acetate buffer (1M) pH 4, 0.4ml of enzyme solution, 0.1ml of colloidal chitin (1mg) in respective composition was used as assay mixture. Colloidal chitin was prepared as per the method of (Roberts and Selitrennikoff, 1988). After 2hour of incubation at 37 ºC the reaction was stopped by centrifugation at 10,000g for 3minutes. An aliquot of supernatant (0.3ml) so obtained was pipetted into a glass reagent tube containing 30µl of potassium phosphate buffer (1M) pH7.1 and incubated with 20µl of (3%w/v) desalted snail gut enzyme Helicase (Sigma) for 1hour, upon which the pH of the reaction mixture was levelled to 8.9 by addition of 70µl of sodium borate buffer (1M) pH 9.8. The reaction mixture was then incubated in a boiling water bath for 3 minutes and then rapidly cooled in an ice water bath. Again incubation was carried out for 20 minutes at 37^{o}C on addition of 2ml of DMAB (ρ-di-methyl-amino-benzaldehyde). Thereof with immediate effect the absorbance value at 585nm was measured using a UV-VIS spectrophotometer. For the protocol N-acetyl glucosamine (GlcNAc) was used as standard (Reissig *et al.*,1959). The enzyme activity was expressed as µg GLcNAc min $^{-1}$ mg $^{-1}$ fresh tissues.

3.25.4. Associated changes in Peroxidase activity.

For the extraction of peroxidase (EC.1.11.1.7) the plant tissue was macerated to powdered form in liquid nitrogen and extracted in 0.1 M Sodium borate buffer (pH 8.8) under cold conditions using ice cubes and flakes with addition of 2 mM ß mercaptoethanol, the homogenate so obtained was centrifuged immediately at 15000 rpm for 20 minutes at 4 ºC. The volume of the supernatant was recorded after carefull collection in a vial, and the same was used for assay or stored at -20^{o}C (Chakraborty *et al*., 1993).

For determination of peroxidase activity, 100µl of freshly prepared crude enzyme extract was added to the reaction mixture containing 1 ml of 0.2 M sodium phosphate buffer (pH 5.4), 100µl of 4mM H_2O_2, 100 µl O-dianisidine (5mg ml-1 methanol) and 1.7ml of distilled water. Peroxidase activity was assayed spectrophotometrically at 460 nm by monitoring the oxidation of O- dianisidine in presence of H_2 O_2 (Chakraborty *et al*., 1993). Specific activity was expressed as the increase in absorbance at 460 nm g^{-1} tissue/ min^{-1}.

3.26. Extraction of total phenol contents from leaves and roots

Phenol was extracted from the fresh leaves and roots following the method of Mahadeven and Sridar, (1982). Initially 1gm of freshly washed and weighed root or leaf samples were cut into small pieces and put in boiling alcohol within a hot water bath for 5-10 minutes with fixed proportion of 4ml alcohol for a gm of tissue. The tubes with the reaction mixture were allowed to boil for 15 minutes after which they were gradually cooled. The samples were the decanted and crushed in mortar and pestle at room temperature. The extract so obtained was passed on through two layers of muslin cloth and filtered through Whatmann filter paper No.1, and the final volume was adjusted with 80% ethanol, which was then subjected to boiling for 15 minutes and then gradual cooling was done. Crushing in mortar and pestle was then done thoroughly at room temperature.

The extract so obtained, was passed through two layers of cheese cloth and then filtered through Whatmann No.1 filter paper. Final volume was adjusted with 80% ethanol, under dark condition to prevent light induced degradation of phenol.

Estimation of total phenols contents

Total phenol content was estimated by Folin Ciocalteau‘s reagent, following the method of Mahadevan and Sridhar (1982). 1ml of test solution in dilutions of 10^{-2} and 10^{-1} (leaf and root) with added 1ml of 1N folin –ciocalteau reagent (1:1) along with 2ml of 20% sodium carbonate solution (Na_2CO_3) was taken in a test tube mixed well and boiled in water bath for exactly 1minute. With gradual cooling dilution of the reaction mixture was done upto 25ml by ddition of distilled water. Absorbance of the solution was measured in a Systronics photometric colorimeter Model 101 at 650 nm. Quantity of total phenol was estimated using caffeic acid as standard.

3.27. Extraction and estimation of total protein contents from leaves and roots

Soluble protein was extracted from the leaves and roots of treated and control plants following the method of Chakraborty *et al*., (1995). The methodology involved freezing the plant tissues in liquid nitrogen and simultaneous grinding in 0.05 mM sodium phosphate buffer (pH 7.2) containing 10 mM $Na_2 S_2 O_5$, 0.5 mM $MgCl_2$ with addition of 2mM PMSF with crushing and centrifugation at 4ºC for 20 min at 12000rpm.The estimation from the supernatant was done following method of Lowry et al. (1951).Bovine serum albumin (BSA) was taken as the standard. The process involved taking supernatant with protein sample 1ml, along with addition of 5ml of alkaline reagent (1ml of 1% $CuSO_4$ and 1ml of 2% sodium potassium tartarate, added to 100ml of 2% $Na_2 CO_3$ in 0.1 NaOH). The reaction mixture was incubated for 15 minutes at room temperature and 0.5 ml of 1N Folin Ciocalteau reagent was added with further incubation for a duration of 15 minutes and then optical density of the solution was measured at 720nm.

3.28. Fluorescence antibody staining and microscopy

Indirect fluorescence staining of cross-section of plant roots and leaves samples were done using FITC labelled goat antirabbit IgG following the method of (Chakraborty and Saha, 1994)

3.28.1. Immunolocalization of Chitinase and Glucanase enzymes by indirect immunofluorescence staining of leaf and root

The steps involved making fine cross section of healthy and treated roots and leaves and subsequent immersion in PBS,ph 7.2.These sections were further treated with normal serum or antiserum in ratio(1:50) in PBS and was allowed to incubate for 1hr at room temperature with cover. The sections were then thoroughly and carefully washed with PBS-Tween pH 7.2 for 15 minutes for about three times. Then sections were subjected under dark conditions to 40µl of diluted (1:40) goat antirabbit IgG conjugated with fluorescence (FITC) and incubation was done for half an hour, under same condition. Sections were again washed thrice with PBS- Tween and were carefully mounted on a grease free slide with 10% glycerol. Fluorescence's of the target sections under treatment were observed using using Leica Leitz Biomed Microscope with fluorescence optics equipped with UV- filter set I-3 and plate photographs were taken.