Chapter 3

3.1. Experimental site
The present study was conducted in the Research Laboratory Department of Biological Sciences’ Sam Higginbottom institutes of Agriculture, Technology and sciences (Deemed University), Allahabad (UP) India.

3.2. Sterilization of Glasswares:
All Glasswares used in this study were of Borosil and corning grade. Sterilization included soaking them in chromic acid for 24 hrs. and rinsing thoroughly with tap water. Finally the Glasswares were rinsed again with distilled water and dried in hot air oven at 180 °C for 3 hrs. culture media as well as cotton plugged glasswares were autoclaved at a pressure of 15 lb / inch² at 121 °C for 15 min. spirit lamp and ethanol were used during the period of inoculation of culture into fresh media.

3.3. Chemical used:
All chemicals used in the present study were produced from Merck, CDH, Hi-Media and were of analytical grade.

3.4. Sampling
The rhizosphere soil samples of different crops were collected and used for the isolation of Heavy metal resistant (PGPR). Total 30 samples viz. 11 from Kanpur and 19 from Sam Higginbotom Institute of Agriculture, Technology & Sciences (SHIATS) (Formerly Allahabad Agriculture Institute) Allahabad district from rhizosphere of Tomato, Chick pea, pigeon pea, Wheat, Pisumsativum were included in the present study.
3.5. ISOLATION OF RHIZOBACTERIA.

10 gram soil sample was added in 90 ml sterile water blanks. The soil suspended in the tube was shaken thoroughly to mix soil particles and get them uniformly dispersed and further dilution $10^{-1}$ to $10^{-5}$ were made through serial dilution technique. One ml aliquots from the dilution ($10^{-5}$) was transferred in sterilized petri dishes and plated on the appropriate medium for isolating different Rhizobacteria viz. nutrient agar for Bacillus, cetrimide for Pseudomonas and YEMA for Rhizobium (Vincent, 1970), three replicates were maintained. the plates were incubated at $35^0$C for 2-4 days in inverted position so that the condensed vapor may not hamper the growth of the isolated bacteria. After incubation bacterial colonies were counted, representative colonies were selected based on distinct types observed according to the morphological characteristics including pigments, colony form elevation and margin, texture, and opacity (Simbert et al., 1981).

3.6 Morphological characterization of isolates

3.6.1. Cell Morphology

For cell shape, size, arrangement, gram staining, motility and capsule staining of 24 hours incubated bacterial cultures were observed.

3.6.2. Motility Test

A hanging drop culture is prepared by placing a very small drop of nutrient broth on a cover slip, then inverting the cover slip over a depression slide so that the bottom of the drop does not make contact with the slide itself.
Vaseline was used, to make a sealed chamber and over served under oil immersion.

### 3.6.3. Gram Staining

The most important differential stain used in bacteriology is the Gram stain, named after Dr. Christian Gram. It divides bacterial cells into two major groups, gram-positive and gram-negative, which makes it an essential tool for classification and differentiation of microorganisms. The Gram stain reaction is based on the difference in the chemical composition of bacterial cell walls. Gram-positive cells have a thick peptidoglycan layer whereas the peptidoglycan layer in gram negative cells is much thinner and surrounded by outer lipid-containing layers. Peptidoglycan is mainly a polysaccharide composed of two chemical subunits found only in the bacterial cell wall. These subunits are N-acetylglucosamine and N-acetylmuramic acid. As adjacent layers of peptidoglycan are formed, they are cross linked by short chains of peptides by means of a transpeptidase enzyme, resulting in the shape and rigidity of the cell wall. Early experiments have shown that if the gram positive cell is denuded of its cell wall by the action of lysozyme or penicillin, the gram-positive cell will stain gram negative.

### 3.6.4. Primary stain

Crystal violet is used first and stains all cells purple. Its function is to impart its color to all cells. In order to establish a colour contrast.

### 3.6.5. Mordant

Gram’s iodine, this reagent is not only a killing agent, but also serves as a mordant a substance that increases the cells’ affinity for a stain. It does this by binding to the primary stain, thus forming an insoluble complex. The
resultant crystal-violet-iodine complex serves to intensify the colour of the stain. At this point, all cells will appear purple black.

3.6.6. Decolorizing agent

Ethyl alcohol, 95% - This reagent serves as a dual function as a protein-dehydrating agent and as a lipid solvent. Its action is determined by two factors, the concentration of lipids and the thickness of peptidoglycan layer in bacterial cell walls. In gram negative cells, the alcohol increases the porosity of the cell wall by dissolving the lipid in the outer layers. Thus the CV-1 complex can be more easily removed from the thinner and less highly cross linked Peptidoglycan layer. Therefore, the washing out effect of the alcohol facilitates the release of the unbound CV-1 complex, leaving the call colorless or unstained. The much thicker peptidoglycan layer in gram positive cells is responsible for the more stringent retention of the CV-1 complex, as the pores are made smaller due to the dehydrating effect of the alcohol. Thus the tightly bound primary stain complex is difficult to remove, and the cells remain purple.

3.6.7. Counterstain

Safranin is used to stain red those cells that have been previously decolorized. Since only gram negative cells undergo decolourization, they may now absorb the counter stain. Gram positive cells retain the purple colour of the primary stain.

3.6.8. Procedure

For Gram-staining a drop of glass-distilled water was placed on clean glass slide. Bacterial culture fresh 24 hours incubated was taken with the help of a sterile lop in a drop of water on the slide. Bacterial culture was mixed in water and smear was spread evenly on slide surface and air-dried. Bacterial smear was thoroughly heat-fixed and stains with crystal violet solution for 1
- 2 minutes, washed with glass-distilled water smear was dipped in 95% alcohol so that the extra stain was removed. Then smear was counterstained with safranine for 30 seconds or 1 minute, washed with glass distained water and air dried and observed under the microscope with an oil immersion lens.

**3.7. Catalase test (Blazevic and Ederer, 1975)**

Nutrient agar slants were inoculated with test organisms and were incubated at 30°C for 24 hours. After incubation the tubes were flooded with one ml of three per cent hydrogen peroxide and observed for production of gas bubbles. The occurrence of gas bubbles was scored positive for catalase activity.

**3.8. Starch hydrolysis**

The ability of the isolates to hydrolyse starch was examined by the procedure of Eckford (1927). Petriplates containing starch agar were inoculated with test cultures and incubated at 30°C for three days. After incubation the plates were flooded with Lugol’s iodine solution and allowed to stand for 15-20 minutes. The clear zone around the colony was considered as positive for the test.

**3.9. Methyl red test**

Test culture containing MR-VP broth were sterilized and inoculated with the test cultures. The tubes were incubated at 28±2°C for 48 hours. After incubation five drops of methyl red indicator was added to each tube and gently shaken. The production of red colour was taken as positive for the test and production of yellow colour was taken as negative for the test.
3.10. Voger – Proskauer test

To the pre-sterilized tubes containing MR-VP broth test cultures were inoculated. The tubes were incubated for 48 hours at 37°C. After incubation tendrops of Barritt’s reagent A was added and gently shaken followed by addition of ten drops of Barritt’s reagent B. The development of rose colour in the broth was taken as positive for the test.

3.11. Hydrogen sulphide production

Bacterial isolates were inoculated to test tubes containing 5 ml of sterile medium and incubated at room temperature 28°C. The test tubes were observed for H₂S production. The formation of black ring in the medium was taken as positive for H₂S production.

3.12. Citrate utilization test

Some microorganisms are capable of utilizing citrate as sole carbon source for metabolism in the presence of enzyme citrase with resulting alkalinity. This test determines whether or not an organism is able to metabolize citrate for energy. Simmon’s citrate agar is a defined medium containing sodium citrate as the sole carbon source and the ammonium ion as the sole nitrogen source. Bacteria can break the conjugate base salt of citrate into organic acids and carbon dioxide. The carbon dioxide can combine with the sodium from the conjugate base salt to from a basic compound, sodium carbonate. A pH indicator, bromothymol blue detects the presence of this compound in the medium and turns form green at neutral pH (6.9) to blue when a pH higher than 7.6 is reached (basic or alkaline). For this test simmon’s citrate agar was prepared, dispensed in test
tubes, autoclaved and tubes were incubated at 37°C. Results were recorded after 24 and 48 hours of incubation.

3.13. Oxidase test
To the trypticasesoyagar plates, overnight culture of the test isolate was spotted and the plates were incubated for 24 hours at 28±2°C. After incubation, two to three drops of tetramethylphenylenediaminedihydrochloride was added to the surface of the growth of test organism. The colour change to maroon was taken as oxidase positive.

3.14. Characterization of Rhizobacteria for Plant Growth Promoting Traits:
All rhizobacterial isolates were characterized for plant growth promoting characteristics based on the standard procedures.

3.14.1. Ammonia Production Test
This test was performed by growing all the isolates in peptone water and incubated at 30°C. After 4 days of incubation 1ml of Nessler's reagent was added to the tube. Appearance of a faint yellow colour indicates small amount of ammonia production and deep yellow brown colour indicates maximum production of ammonia.

3.14.2. Indole Acetic Acid (IAA) production test
The bacteria were cultured in flasks containing 20ml of LB medium (Appendix-) supplemented with 0.2 -0.5mg /ml of tryptophan. After 4 days of incubation, 1ml of cell suspension was transferred into a tube and mixed vigorously with 2ml of Salkowski's reagent and few drops of Orthophosphoric Acid and allowed to stand at room temperature for min,
after which pink colour was developed in the cell suspension which indicates the IAA production.

3.14.3. Phosphate Solubilization Test:

Bacterial isolates were tested for Phosphate Solubilization ability on Pikovaskaya medium (Pikovaskaya, 1948) incorporated with TricalciumPhosphate. Phosphatesolubilization was indicated by a formation of clear halo zone around the bacterial growth after 3 days of incubation.

3.14.4. HCN Production Test

The isolates were streaked on King's B medium amended with 4.4g/l of Glycerin. The plates were covered with sterile filter paper impregnated with 0.5% Picric acid in 2% Sodium Carbonate sealed with Para film and incubated for 4 days. Development of yellow colour on the filter paper indicates the positive results.

3.15. Physico-chemical analysis of soil:

3.15.1. Sampling and analysis of soil:

The soil samples were collected with the help of a soil auger. The representative soil samples were transferred into tight polythene bags and brought into laboratory. The soil samples were dried at 105° C for 24 hrs. in the hot air oven and crushed to pass through a 2mm nylon sieve.

Table: 3.1. List of physico-chemical parameters and methods employed for the analysis of soil samples:
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Method Employed</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH (1:2)</td>
<td>Digital pH meter (Model PR 8404) (M.L.Jackson 1973)</td>
</tr>
<tr>
<td>EC (1:2) (dSm⁻¹)</td>
<td>ELECTRICAL Conductivity Meter (CM 827)</td>
</tr>
<tr>
<td>Organic Carbon (%)</td>
<td>Walkley and Black Method (1934)</td>
</tr>
<tr>
<td>Available Nitrogen (Kg ha⁻¹)</td>
<td>Subbiah and Asija (1966)</td>
</tr>
<tr>
<td>Available Phosphorous (Kg ha⁻¹)</td>
<td>Olsen spectrophotometer Method (Olsen et al., 1954)</td>
</tr>
<tr>
<td>Available Potassium (Kg ha⁻¹)</td>
<td>Flame photometric Method (Jackson 1973)</td>
</tr>
</tbody>
</table>

Soil samples were analyzed for physicochemical parameters like pH, Conductivity, total organic carbon, total nitrogen, available potassium, available phosphorous, etc.

3.15.2. Determination of pH (1:2) in soil:

20 gm soil in 40 ml distilled water and stirred the suspension with a glass rod intermittently for 30 minutes. Soil particles were allowed to settle down. Standardized the pH meter by immersing the electrode in the buffer solution of known pH 9.2 and 7.0. After standardization pH of soil was recorded.

3.15.3. Determination of electrical conductivity (dsm⁻¹) in soil:

20 gm soil in 40 ml distilled water and stirred the suspension with a glass rod intermittently for 30 minutes. Wait until the particle of
soil settled down. Then the instrument was calibrated or checked and then EC of soil was recorded.

3.15.4. Determination of organic carbon (%) in soil:

1 gm of soil was taken and transferred to a 500ml conical flask. Then 10ml of 1N K$_2$Cr$_2$O$_7$ was added to it with a pipette and mixed the content by swirling the conical flask. Further, 20 ml of conc. H$_2$SO$_4$ was added slowly along the inner wall of the flask. The flask was rotated gently to ensure complete contact of the reagents with the soil. The flask was kept on wire gauze for 30 minutes to allowed the completion of the oxidation of organic carbon. After 30 minutes of oxidation of organic carbon, 100ml of distilled water was added, followed by 10ml of 85% H$_3$PO$_4$ and 2ml of diphenylamine indicator (0.5%) into the flask. The solution was titrated by ferrous ammonium sulphate until the solution colour changed from the blue violet to green. Finally 0.5 ml of 1N K$_2$Cr$_2$O$_7$ was added and the solution was titrated again by ferrous ammonium sulphate. The Burette reading was measured for final reading. A blank sample was also run similarly.

3.15.5. Determination of available nitrogen (kg g$^{-1}$) in soil:

1 gm of paraffin with 20 gm soil sample (<2mm) was transferred into 800ml Kjeldahl flask. The distillation apparatus was set and condenser tube was dipped into 250 ml of conical flask containing 2% H$_3$PO$_4$. There after 100 ml of 0.32% KMnO$_4$ solution (freshly prepared) was added followed by 100 ml of 2.5% NaOH solution. Then the contents were boiled on the heating unit. 110 ml distillate was collected into 250 ml conical flask containing boric acid. NH$_4^+$ was titrated with the help of burette formed with 0.02N HCL to the light pink colour from green. Similarly blank distillate was run a containing
40 ml of 2% boric acid containing mixed indicator. The Burette reading was measured for final reading.

### 3.15.6. Determination of Available Phosphorous in soil:

**Reagents required:**

- **0.5 M Na HCO₃:** 42 gram of Na HCO₃ was dissolved in about 500 ml of distilled water and the volume was made up one litre. The pH was adjusted to 8.5.

- **Reagent A:** 12gm of ammonium molybdate was dissolved in about 250 ml of warm distilled water. In a separate beaker 0.291 gm of antimony potassium tartarate was dissolved in 100 ml of warm distilled water. After cooling both the solutions were mixed.

- **5 N H₂SO₄:** 140 ml of conc. H₂SO₄ was diluted to one litre with the help of distilled water.

- After cooling ammonium molybdate and antimony potassium tartarate solution was mixed to 5 N H₂SO₄ in 2 litre volumetric flask and volume was made 2 litre.

- **Paranitrophenol Indicator:** p- nitrophenol (0.5 gm) was dissolved in 100 ml of distilled water.
• Reagent B (Ascorbic acid solution): 1.056 gm of ascorbic acid was dissolved in 200 ml of the molybdate solution (Reagent A) and was mixed properly.

2.5 gm of soil was taken in 100 ml conical flask and 50 ml of Olsen’s reagent was added with a pinch of Dargo G-60 (Charcoal powder) and shaken for 30 minutes on a mechanical shaker and similarly a blank was also run without soil. The solution was filtered in reagent bottle and suitable amount of aliquot (2-5ml) was pipetted out in 25/50 ml volumetric flask. A drop of p-nitrophenol was added, yellow colour develops, neutralised the yellow colour by adding 5N sulphuric acid and 2 ml of ascorbic acid solution (Reagent B).

3.15.7. Determination of available potassium (kg ha⁻¹) in soil:

Reagents:
• Neutral normal ammonium acetate: Dissolve 77.08 gm of ammonium acetate in about 800 ml of distilled water in one litre volumetric flask. Adjust the pH to 7.0 with dilute acetic acid and ammonia solution and make the volume to one litre.

• Standard K solution: Prepare 1000 ppm K solution by dissolving 1.908 gm of dried KCl salt in 1000 ml distilled water.

• Working standard K solution: Dilute 0.5 ml, 1 ml, 1.5 ml and 2.0 ml of 1000 ppm K solution to 100 ml to get 5, 10, 15 and 20 ppm K solution.

Procedure:
5 gm of soil was taken in 100 ml conical flask. 25 ml of ammonium acetate solution was added and kept for five minutes on mechanical shaker. The solution was filtered. K concentration was measured in the filtrate using flame photometer after standardization of the instrument with the help of potassium standard solution.

Table 3.2 Physicochemical analysis of soil samples.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Parameter</th>
<th>Observed value</th>
<th>Method Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>pH</td>
<td>6.5-7.63</td>
<td>pH meter (Jackson, 1967)</td>
</tr>
<tr>
<td>2.</td>
<td>Electrical conductivity (dSm⁻¹)</td>
<td>0.30</td>
<td>EC bridge (Jackson, 1973)</td>
</tr>
<tr>
<td>3.</td>
<td>Organic carbon (%)</td>
<td>0.82%</td>
<td>Walkely and Black’s wet oxidation method (Jackson, 1967)</td>
</tr>
<tr>
<td>4.</td>
<td>Nitrogen (kg/ha)</td>
<td>338.64</td>
<td>Alkaline permanganate method (Subbaiah and Asija, 1966)</td>
</tr>
</tbody>
</table>
5. Phosphorus (kg/ha) | 33.42 | Olsen’s method *(Muhr et al., 1965)* 

6. Potassium (kg/ha) | 357.32 | Flame photometer method *(Stanford and English, 1949)* 

<table>
<thead>
<tr>
<th>Source for Isolation</th>
<th>Bacterial Isolates (cfu × 10^6 /g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient Agar Medium</td>
<td>1.6×10^6</td>
</tr>
<tr>
<td>King's B medium</td>
<td>1.9×10^6</td>
</tr>
<tr>
<td>Yeast extract mannitolAgar</td>
<td>1.7×10^6</td>
</tr>
</tbody>
</table>

**3.16. Determination of heavy metal tolerance of bacterial isolates**

Tolerance to heavy metal ions was determined by Minimal Inhibitory Concentration (MIC) technique. Stock solution of heavy metals was prepared in a concentration of 200µg/ml and sterilized separately. Nutrient Agar media was prepared in conical flasks and sterilized in autoclave at 15 lb /
in² for 15 min. Nutrient Agar plates were amended with appropriate concentration of heavy metals and test organisms were inoculated with sterile inoculation needle. Inoculated plates were incubated for 24-48 hrs. The minimal inhibitory concentration of the metal inhibiting the complete growth was taken as MIC. Metals used were namely Cr, Pb, Hg, Cd, Zn, Co and Cu at different concentrations (25-200µg/ml).

3.17. Morphological Analysis of tomato seedlings:

Surface sterilized and uniform size tomato (*Lycopersicon esculentum* L.) seeds were inoculated with 30 selected rhizobacterial isolates viz. YSY-1 to YSY-30 were placed in petridishes. Uninoculated seeds were maintained as control treated. After 3, 5, and 7 days seedlings were taken out for various studies like root and shoot length and the data were recorded.

For determining effect of rhizobacteria on root and shoot length. Seeds were inoculated with 50 ppm concentration of K$_2$Cr$_2$O$_7$ (Cr). After 3, 5, and 7 days seedlings were taken out for various studies like root and shoot length and the data were recorded.

To determine combined effect of heavy metal (Cr) and rhizobacteria, seeds were inoculated with 50 ppm concentration of K$_2$Cr$_2$O$_7$ along with selected rhizobacterial strains. After 3, 5, and 7 days after germination were taken out for various studies like root and shoot length and the data were recorded.

3.18. Biochemical studies of plant materials
3.18.1. Estimation of chlorophyll

The estimation of chlorophyll was done by using dimethyl sulfoxide (DMSO) extraction procedure. Fresh tomato leaves were collected at random and leaves were chopped into fine pieces. 50 mg sample from these chopped leaves were added in replicated tubes each containing 10 ml dimethylsulphoxide (DMSO).

The tubes containing leaf pieces and DMSO were incubated at 65°C for 3 h in an oven by providing gentle shake twice. After complete extraction, clear supernatants were used for measuring the absorbance with the help of a spectrophotometer against DMSO blank. The chlorophyll a, chlorophyll b and the total chlorophyll content were calculated according to the formula given below on mg g⁻¹ fresh weight of leaf tissue basis (Hiscox and Isralesham, 1979).

a. Chlorophyll a (mg/g fresh weight)

\[
V = \frac{(12.7 \times A_{663} - 2.63 \times A_{645})}{1000 \times W}
\]

b. Chlorophyll b (mg/g fresh weight)

\[
V = \frac{(22.9 \times A_{645} - 4.48 \times A_{663})}{1000 \times W}
\]

b. Total Chlorophyll (mg/g fresh weight)

\[
V = \frac{(20.2 \times A_{645} + 8.02 \times A_{663})}{1000 \times W}
\]

3.18.2 Protein estimation

Protein was estimated from plant leaves using Lowry’s method (Lowry et al., 1951).

3.18.3 Procedure
Estimation of protein was done by pipetting out 50 μl supernatant containing proteins into test tubes in replicates of three and the total volume was made up to 1 ml. A tube with 1 ml distilled water served as a blank. 3 ml reagent C was added to each tube including the blank and after proper mixing the solutions were allowed to stand for 30 min then 0.5 ml reagent D was added and after mixing, the tubes were left at room temperature in the dark for 60 min. Blue colour developed in the solution. The absorbance was taken at 660 nm in UV-visible spectrophotometer. With the help of the standard curve the amount of protein in rice leaves was estimated.

3.18.4 Sample preparation for Total soluble protein estimation (Laemmli, 1970)

0.1 g of fresh tomato leaves were cut into fine pieces and transferred into an eppendorf. 1 ml extraction buffer containing 9:1 LSB and 10% mercaptoethanol (900 μl of LSB + 100 μl of 10% mercaptoethanol) was added to each eppendorf. The eppendorfs were then transferred to water bath at 100°C for 5 min. The eppendorfs were then taken out and kept on ice. The samples were centrifuged at 5,000 g for 1 min at 0°C. Then the supernatant was collected. This supernatant was used for protein quantization.