CHAPTER 4
MATERIALS AND METHODS

In the present investigation laboratory studies were conducted at Dr. D. Y. Patil Biotechnology and Bioinformatics Institute, Dr. D.Y. Patil Vidyapeeth Pune. The details of materials used and methodology followed during the course of investigation is described in this chapter.

4.1 Bacterial strains and plasmid:

The slow growing *Rhizobium japonicum* AVR was used as reference strain in this experiment. *Rhizobium japonicum* AVR1 produced from wild type as spontaneous rifampicin resistance mutant. *E.coli* S17 harboring suicide plasmid pKO3 containing Tn3 which is used for bacterial mating experiment.

Table 4-1: Bacterial strains, plasmid and relevant characteristics

<table>
<thead>
<tr>
<th>Strains or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhizobium japonicum</em> AVR</td>
<td>Wild type local isolate from the farm</td>
<td>This work</td>
</tr>
<tr>
<td><em>Rhizobium japonicum</em> AVR1</td>
<td>Derivative of wild type, Rif(^{r})</td>
<td>This work</td>
</tr>
<tr>
<td><em>E.coli</em> S17 (pko3)</td>
<td>Carrying Tn3 suicide vector</td>
<td>Bangalore</td>
</tr>
<tr>
<td></td>
<td>Amp(^{r}), Chl(^{r})</td>
<td>GeNei, India</td>
</tr>
</tbody>
</table>

4.2 Bacterial cultural media:

4.2.1 Yeast Extract Mannitol Agar plates:

YEMA medium is suitable medium for *Rhizobium* (Somasegaran and Hoben 1994) 1000 ml of YEMA medium is prepared by dissolving following contents, 1 g of yeast extract powder, Mannitol 10 g, dipotassium phosphate 0.5 g, magnesium sulphate 0.2, sodium chloride 0.1 in 1000 ml water and pH is adjusted to 7.0.
4.2.2 YEMA+Congored plates:
Congo red dye is added in YEMA medium for differentiation of Agrobacterium and Rhizobium (Somasegaran and Hoben). 0.025 Congo red powder is added in 1000 ml of YEMA medium.

4.2.3 Luria-Bertani agar plates:

40 g of LB agar powder (Himedia) was dissolved in 1 litter of sterile water and autoclaved at 121°C for 15 minutes and then cooled at 55°C. Antibiotics chloramphenicol (20 µg/ml) andampicillin (100 µg/ml) was added quickly prior to pouring in to Petri dishes. Plates permitted to set at room temperature, dried at 37°C overnight and stored at 4°C for later use.

4.2.4 Luria-Bertani broth:

25 g LB broth powder (Himedia) was dissolved in 1 litre of sterile water and autoclaved at 121°C for 15 minutes, and medium was stored at 37°C for 2-3 days. If medium was not used quickly, it was prepared in aliquots of 50mL and stored at 4°C until required.

4.2.5 M9 Agar medium:

M9 medium is used for screening of transconjugate (Neeraj et al 2009). For preparation of M9 medium first stock solutions were prepared. First stock is M9 salts dissolve Na₂HPO₄ 60g, KH₂PO₄ 30g, NaCl 5g, NH₄Cl 10g into 1 litre of water adjust pH 7.4 with 10 M NaOH and then sterilize. Second stock is autoclaved 1M MgSO₄. Third stock solution is 1M CaCl₂ autoclaved and fourth stock is filter sterilize 40% (W/V) glucose. Autoclave 900 mL of water and 20g of agar powder let it cool and then aseptically add M9 salts, 100 mL 2mL 1M MgSO₄, 0.1 M CaCl₂, 5 mL of 40% glucose.
4.2.6 M9 broth medium:
For M9 broth all stock solutions are same as M9 agar, water is added instead of agar.

4.2.7 Antibiotic preparation:
Antibiotics were used at subsequent concentrations when at work with *Rhizobium japonicum*; rifampicin (50mg/mL) was prepared in DMSO. Ampicillin and Chloramphenicol stocks were made in distilled water at a concentration of (100µg/mL) respectively when working with *E.coli*. The antibiotic stocks were stored at -80°C until required. The antibiotics and chemicals obtained from SRL Pvt. Ltd. (India).

4.2.8 Glycerol stocks of bacteria:
Glycerol stocks were prepared by addition of 500µL of late-log phase bacterial cultures (O.D<sub>600</sub> 0.8-1.0) to the same volume of sterile 50% glycerol, and stored at -20°C until required.

4.2.9 Isolation of rhizobia from mung bean nodules
Nodules were collected from field. Immerse whole unharmed nodules for 10 sec. in 95% ethanol; transfer to a 2.5-3% sodium hypochlorite, and soak for 2-3 minutes as designated by Somasegaran and Hoben (1994). Surface sterilized nodules were taken in a sterile petri dish, with the help of sterile forceps and scaple nodules were cut in to small pieces. These pieces were suspended in to a sterile test tube containing 10 mL of sterile water and macerated gently with a sterile glass rod. When the suspension became slightly turbid due to bacterial cells, it was serially diluted up to 10<sup>-6</sup> with 9 mL sterile distilled water blanks. 0.1 mL of 10<sup>-6</sup> diluted suspension was spread on sterile YEMA + Congo red plates. The medium autoclaved at 121°C at 15 PSI for 15 min. The inoculated plates were incubated at 28°C for 144 hr and observed for the development of well separated typical, white coloured bacterial colonies resembling *Rhizobium* species.
4.2.10 Purification and maintenance of culture

The plates with separate bacterial colonies were selected. White coloured colonies were selected on the Congo red + YEMA surface, because there are chances of presence of closely related genus Agrobacterium. Rhizobial colonies weakly absorb Congo red and remain white in colour while, Agrobacterium absorb Congo red and remain red in colour (Somasegaran and Hoben.1994). The selected colonies were purified by four quadrant streak method on yeast extract Mannitol agar plates by incubating at 28°C for 144 hr. All the colonies were transferred on to the slants containing yeast extract Mannitol agar medium for short term preservation. All purified isolates were stored at glycerol stock at -80°C for long term preservation; it also serves as stock culture for further studies.

One isolate, showing typical pinkish white colour were selected from the growth on petri dishes which were designated as AVR.

4.3 Characterization of culture

The identification of isolate was carried out by nodulation test, morphological, biochemical and physiological characteristics studies as per standard microbiological procedure.

4.3.1 Nodulation test

The nodulation ability of isolate was determined by inoculating seeds of mung bean with culture suspension of Rhizobium japonicum. Three seeds of Latika variety of mung bean were obtained and surface sterilized with 3% sodium hypochlorite as described by Somasegaran and Hoben. Three seeds each placed in 10 pots and overnight culture of Rhizobium japonicum was used for inoculation. Sterilized soil and water used for nodulation test, after 30 days nodulation was observed in these pots. Control pots are
without inoculum. After 30 days pink coloured nodules were seen on root system of inoculated mung bean plant. The organism was reisolated from nodule to compare with their original culture.

4.3.2 Morphological characteristics:

The morphological characteristics of the isolate such as cell shape, size, colour and gram reaction were carried out as per the standard procedure described by Somasegaran and Hoben (1994).

4.3.3 Physiological Characteristics:

4.3.3.1 Optimum temperature for growth of isolate:

The study was conducted to find out optimum temperature for growth of bacterial isolate forming nodules on root system of mung bean plant.

The bacterial stock culture was revived in Yeast Extract Mannitol Agar medium. The culture was prepared by inoculating a loopful of bacterial culture from stock culture to pre-sterilized 100mL YEM broth contained in 250 mL conical flask and incubated for 144 hr at 280C. 100 mL of YEM broth containing in 200 mL Erlenmeyer flask. The inoculated flasks were incubated at temperatures viz, 5,10,15,20,25,28,30,35,400C FOR 144 hr. Observations were recorded for the growth of bacterial colonies in the inoculate flasks kept at various temperature levels. Bacterial growth was observed by optical density value at 600 nm by spectronic 200 spectrometer.

4.3.3.2 Determination of optimum pH for growth of Isolate:

The effect of hydrogen ion concentrate on growth of bacterial isolate was evaluated by altering the pH of the YEMA. The pH was adjusted to 3, 4, 5, 6, 7, 8
and 9 using appropriate buffers. A 72 hr old bacterial culture \(2.8 \times 10^6 \text{ cfu/mL}\) was transferred in 100 mL broth containing in 200 mL Erlenmeyer flask incubated at 280C for 72 hr. After the incubation period, observations were recorded for the growth of the bacterium in media having different pH levels. Results were recorded by observing their O.D value at nm by spectronic 200 spectrometer.

4.3.4 **Biochemical characteristics:**

The biochemical characters such as bromothymol blue, glucose peptone agar and gelatine hydrolysis by the isolates were studied.

4.3.4.1 **Bromothymol blue assay:**

The *Rhizobium* were categorized as fast growers when medium turn yellow and slow growers when medium turned blue based on their reaction on YEMA added with bromothymol blue (0.5 g/L ml ethanol stock solution, add 5 mL stock/litre YMA) a described by Somasegaran and Hoben (1994).

4.3.4.2 **GPA (Glucose peptone agar assay):**

GPA assay used to determine ability of *Rhizobium* to utilize glucose as a sole source carbon for its growth. GPA medium (40 g/L glucose, 5 g/L peptone, 15 g/L agar, pH 7.0) inoculated with culture, incubated and growth was recorded.

4.3.4.3 **Gelatine liquefaction:**

The test was made to decide ability of organism to produce gelatinase. Degradation of gelatine shows the presence of gelatinase (Aneja, 2003). The actively grown culture inoculated in gelatine medium (5 g/L peptone, 3 g/L beef extract, 15 g/L agar, 12 g/L gelatine) and grown for 48 hrs. On exposing the growing culture to low temperature treatment at 40C for 30 min, the cultures which produce
gelatinase remains liquefied though others due to attendance of gelatine becomes solid.

4.3.4.4 Hydrolysis of starch:

The test was performed so as to determine ability of *Rhizobium* to use starch as a carbon source (de oliveria, 2007). Starch agar media (5 g/L peptone, 3 g/L beef extract, 15 g/L agar, 2 g/L potato starch pH 7.0) were incubated with culture and examined. Iodine test was used to govern ability of microorganism to use starch. Drops of iodine solution (0.1N) were spread on 48 h old culture grown on petri plates. Development of blue colour indicated non-utilization of starch and vice versa.

4.4 Production of spontaneous rifampicin resistant mutants:

The spontaneous rifampicin resistant mutants of *Rhizobium japonicum* were produced by streaking wild type culture suspension of 2.8 x 10⁶ on YEMA medium containing rifampicin (100 µg/ml) and were maintained on the same medium containing rifampicin (25µg/ml). One isolate randomly selected from the growth on petri dishes containing rifampicin and designated as AVR 1 and subjected to nodulation test.

4.4.1 Bacterial mating

Mating between *Rhizobium japonicum* and *E.coli* were conducted by method of Khetmalas (1996).Cells of donor were harvested by centrifugation at the exponential growth phase and recipient at stationary phase. Both cells were washed three times with10 mM MgSO₄ and mixed at a ratio of 1:1. Here donor cell is *E.coli* and recipient cell is *Rhizobium japonicum*. Then 100µL of cell suspension was transferred to 0.45 µm pore size; 25-mm in diameter filter placed on mating medium. Controls were kept as individual filtered portions of the above cells, after incubation at 8 hrs at 30⁰C; the filter
was transferred to 2.0 ml of L.B medium, diluted and plated. The transposition frequency was defined as ratio of colonies scored on selective medium compared with the number of colonies scored on non-selective medium after mating. Colonies of 800 transconjugants were produced by bacterial mating and these 800 transconjugants were designated as AVR 01 to AVR 0800. All the designated mutants were subjected for nodulation test.

4.4.2 Reisolation of bacteria:

Bacteria were reisolated from nodules inoculated with mutants and plated on M9 medium with rifampicin (100 µg/ml) and ampicillin (100 µg/ml). Reisolated mutants were compared with original mutants.

4.4.3 Estimation of nitrogenase activity:

4.4.3.1 Acetylene reduction method:

The nitrogenase enzyme has a great role in BNF. Nitrogenase is produced by bacteroid in the nodules. Throughout the reduction process molecular nitrogen is changed to NH₃ through a sequence of stages involving enzyme (s) and ATP. Though molecular nitrogen is the natural substrate for nitrogenase, other triple bonded “nitrogen –analogues” like nitrous oxide (N=N-O) acetylene (HC=CH), cyanide (H-C=N), and methyl isocyanide (CH₃-N=C) can also undergo reaction facilitated by nitrogenase complex, root nodules of legumes are open to 10% of acetylene in air mixture and incubated at room temperature for 1 hr. The ethylene (C₂H₄) produced is calculated by gas chromatography, though it is sensitive method for assaying nitrogenase activity. The whole process of nitrogenase activity was carried out by method as described by Somasegaran and Hoben (1994) at Vasantdada Sugar Institute, Manjri, Br. Pune.
4.4.3.2 The Gas Chromatograph

A gas chromatograph through (FID) is usually used in the assay. A steel column of 3 m long and 1mm in diameter, is filled with molecular sieve material, usually Porpak media. Porpak is a porous polymer composed of ethylvinylbenzene cross-linked with divinylbenzene to form a uniform structure of distinct pore size. It is obtainable in dissimilar mesh size and allows good separation of C₂H₂ and C₂H₄. Different gases have different retention times in the column; therefore gas chromatograph-recorder will trace out there peak in order of emergence. In this assay Perkin Elmer Gas chromatography with dual porpak N column of 2.0 M length with standard flame ionization detector was used. After incubation of root nodule with acetylene in air, 0.5 ml of the gas mixture was fractionated by injection into a gas chromatograph FID.

Figure 4-1:- Gas Chromatograph Instrument
The nitrogen fixed from atmosphere was estimated based on the peak area of ethylene as a result of acetylene reduction by nitrogenase, using following equation of Bender and Rolf (1985).

\[
\text{nM C}_2\text{H}_4 \text{ h}^{-1} \text{ mg}^{-1} \text{nodules} = \frac{\text{concentration of ethylene} \times \text{area of ethylene} \times \text{volume} \times 2}{\text{concentration of standard} \times \text{incubation time} \times \text{mg nodules, and using a ratio of } 4 \text{ n mol C}_2\text{H}_4 \text{ produced for } 1 \text{ mol N}_2 \text{ fixed}}.
\]

4.5 Extraction of total genomic DNA:

4.5.1 Bacterial DNA isolation:

Total bacterial DNA was isolated using QIAGEN DNeasy Kit (Cat#56404) as per the manufacturer’s instruction. Briefly the protocol was as below: single bacterial colonies (4 to 5) were picked using sterile pipette tip and suspended in PBS buffer. Cells were washed by centrifugation at 4000 RPM for 10 min. Pellet was suspended in 180 µL ATL buffer. Cell suspension was incubated and further processed using spin column protocol. DNA was eluted in 50 µL Elution buffer.

4.5.2 Agarose gel electrophoresis of Bacterial DNA:

Agarose gel electrophoresis of the genomic DNA was performed using 1.0% (w/v) agarose gel using standard 0.5X TBE gel electrophoresis buffer. PCR product of 5.0 µL was mixed with 1 µL of 6X Gel loading dye. The DNA molecules were resolved at 5V/cm until the tracking dye is 2/3 distance away from the lane within the gel. Bands were detected under a UV Trans illuminator. Gel images were recorded using BIO-RAD GelDoc-XR gel documentation system.
4.5.3 PCR amplification:

As per the background information about the cultures, these cultures were Tn3 Transposon mutagenesis induced cultures and are required to be confirmed by molecular tools and identify the putative site of transposon mutagenesis. The presence of transposable elements on bacterial DNA can be determined by PCR amplification using Transposon specific primers. PCR primers as per table below were selected and provided as reference primers for this experiment. These primers were adapted from previous research (Ferreira et al. 2004).

**Table 4-2:- Primer pair used for PCR Amplification:**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Name</th>
<th>Primer sequence</th>
<th>Length</th>
<th>Amplicon Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNP1</td>
<td>TNP-F1</td>
<td>GCCGCCAAGGATGTGCTCGAC</td>
<td>21</td>
<td>500-600 bp</td>
</tr>
<tr>
<td></td>
<td>TNP-R1</td>
<td>CGCGGGTATCGGAAGAAAACA</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

PCR mix was prepared as per composition provided in table below. For each primer pair three reactions were performed, two for DNA samples and one negative control. Final volume of each reaction was 25.0 µL. Genomic DNA was added later to each tube.

**Table 4-3:- Composition of contents used for PCR reaction**

<table>
<thead>
<tr>
<th>Materials</th>
<th>Volume (µl)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomic DNA</td>
<td>2.00</td>
<td>100-200 ng</td>
</tr>
<tr>
<td>10X PCR buffer</td>
<td>2.50</td>
<td>1X</td>
</tr>
<tr>
<td>50mM MgCl2</td>
<td>0.75</td>
<td>1.5mM</td>
</tr>
<tr>
<td>0.5mM dNTP Mix</td>
<td>0.50</td>
<td>0.1 mM each dNTP</td>
</tr>
<tr>
<td>10 pmole primer solution</td>
<td>1.00</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>(TNPF1, TNPR1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taq DNA polymerase (5.0)</td>
<td>0.20</td>
<td>1.0 unit/reaction</td>
</tr>
<tr>
<td>Nuclease Free water</td>
<td>18.05</td>
<td></td>
</tr>
</tbody>
</table>

The reaction was performed in Applied Biosystems 2720 thermal cycler

**Table 4-4:- Stages used in Applied Biosystem Cycler**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature (°C)</th>
<th>Time (min: sec)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>5:00</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>1:00</td>
<td>35</td>
</tr>
<tr>
<td>Annealing</td>
<td>51</td>
<td>1:00</td>
<td>cycles</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>1:00</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>10:00</td>
<td></td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>Until use</td>
<td></td>
</tr>
</tbody>
</table>

**4.5.4 Agarose gel electrophoresis of PCR products:**

Agarose gel electrophoresis of the PCR products was performed using 2.0% (w/v) agarose gel using standard 0.5X TBE gel electrophoresis buffer. Agarose gel (2%w/v) spiked with Ethidium bromide at a final concentration of 0.5 µg/ml was prepared in 0.5X TBE buffer. 5.0 µL of PCR product was mixed with 1µ of 6X Gel loading dye. 5 µL of gScale 100-1000 bp size standard was loaded in one lane for confirmation of size of the amplicon using reference ladder. The DNA molecules were resolved at 5V/cm until the tracking dye is 2/3. Bands detected under a UV Trans illuminator and images recorded using BIO-RAD GelDoc-XR gel documentation system. The PCR product of size between 500 to 600 bp was expected to be generated through this reaction.
4.6 DNA sequencing of amplified PCR products:

4.6.1 ExoSAP:

Purification of PCR products: Before sequencing of PCR amplicons, it is necessary to remove unused dNTPs and primers from the reaction mixture. Five μl of a post-PCR reaction product was mixed with 2 μl of ExoSAP-IT reagent for a combined 7 μl reaction volume. Reaction was incubated at 37°C for 15 minutes to degrade remaining primers and nucleotides. The reaction was then inactivated by incubation at 80°C for 15 minutes. The PCR product is now proceed ready for DNA sequencing.

4.6.2 Cycle Sequencing:

ExoSAP purified PCR products (50ng) were used for DNA sequencing. ABI BigDye® Terminator v3.1 Cycle Sequencing reaction kit (Applied Biosystems, USA), was used. Following is the sequencing reaction composition.

Table 4-5:- Sequencing Reaction composition:

<table>
<thead>
<tr>
<th>Materials for 10 uL sequencing</th>
<th>Volume (µl)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product DNA</td>
<td>3.00</td>
<td>50-100ng</td>
</tr>
<tr>
<td>Sequencing Buffer</td>
<td>1.80</td>
<td>1X</td>
</tr>
<tr>
<td>RR-100 (Ready Reaction Mix)</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>10 picomole sequencing primer</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Nuclease Free water</td>
<td>3.7</td>
<td></td>
</tr>
</tbody>
</table>

Sequencing reaction was run in 2720 Thermal Cycler (Thermo Fisher) in standard BDT cycle sequencing program: 25 cycles of [96°C for 10 sec, 50°C for 10 sec, 60°C for 4 min], then ramp to 4°C.
4.6.3 **Clean up:**

Cycle sequencing PCR products were then purified by EDTA-Ethanol precipitation protocol. The cleaned up sequencing products were dried at 37° C for 30 minutes and then dissolved in HiDi-Formamide solution (10 µL). The reaction tubes were then subjected to denaturation at 95° C for 3 min and snap chilling at 4° C. These products were loaded on Applied Biosystems DNA sequencing machine (3130 Genetic Analyzer) for Sanger sequencing. Softwares used: Sequencing Analysis 5.1; ChromasPro v3.1.

4.6.4 **BLAST and alignment analysis:**

DNA sequences were generated in .ab1 and FASTA format in sequencing machine and further analyzed by Sequencing Analysis 5.1 software. Using forward and reverse strand sequences a contig of trimmed sequence was generated. For each sample one FASTA sequence was thus generated and further analyzed.

4.6.4.1 **BLAST analysis:**

Sequencing similarity of the samples sequence with Genbank Database sequences was analysed by nucleotide BLAST on *Bradyrhizobium japonicum* genomic sequence.

4.6.4.2 **Clustal W:**

Clustal W alignment was used for comparing difference sequences and finding out percentage similarity between them.