# Chapter 2

# Materials and Methods

## 2.1: Bacterial strains / Plasmids

All the wild type and genetically modified *E. coli* and *Pseudomonas* strains are listed in **Table 2.1 and 2.2**. The plasmids used in the present study and their restriction maps are given in **Table 2.3** and **Fig. 2.1**. *E. coli* JM101 was used for all the standard molecular biology experiments wherever required. The *ppc* mutant strain, *E. coli* JWK3928, was a generous gift from NARA Institute of Science and Technology (Japan) due to kind recommendation of Prof. H. Mori. The *glt*A (citrate synthase gene) mutant of *E. coli* was obtained from *E. coli* Genetic Stock Center (CGSC), Yale University, U.S.A.

**Table 2.1: List of *E. coli* strains used in the present study.**

Ap=Ampicillin; Km=Kanamycin; Tc=Tetracycline; Str=Streptomycin; Tmp =Trimethoprim; r = resistant

| Bacterial Strains | Genotype | Reference |
|---|---|---|
| *E. coli* JM101 | F′ *traD36 proA+B+ lacIq* Δ*(lacZ) M15/* Δ*(lac-proAB) glnV thi* | Sambrook and Russell, 2001 |
| *E. coli* S17.1 | *thi pro hsdR recA RP4-2* (Tet::Mu) (Km::Tn7); Tmpr | Simon et al.,1983 |
| JM101 (pAB3) | *E. coli* JM101 with pAB3 plasmid; $Ap^r$, $Tc^r$ | Buch et al., 2008 |
| JM101 (pAB4) | *E. coli* JM101 with pAB4 plasmid; $Ap^r$, $Tc^r$ | Buch et al., 2008 |
| JM101 (pAB7) | *E. coli* JM101 with pAB7 plasmid; $Ap^r$, $Km^r$ | Buch et al., 2008 |
| JM101 (pAB8) | *E. coli* JM101 with pAB8 plasmid; $Ap^r$, $Km^r$ | Buch et al., 2008 |
| S17.1 (pAB3) | *E. coli* S17.1 with pAB3 plasmid; $Tmp^r$, $Ap^r$, $Tc^r$ | Chapter 3 |
| S17.1 (pAB4) | *E. coli* S17.1 with pAB4 plasmid; $Tmp^r$, $Ap^r$, $Tc^r$ | Chapter 3 |
| S17.1 (pAB7) | *E. coli* S17.1 with pAB7 plasmid; $Tmp^r$, $Ap^r$, $Tc^r$ | Chapter 4 |
| S17.1 (pAB8) | *E. coli* S17.1 with pAB8 plasmid; $Tmp^r$, $Ap^r$, $Tc^r$ | Chapter 4 |

| | | |
|---|---|---|
| DH1OB | Used to maintain plasmids for routine use | Invitrogen, USA |

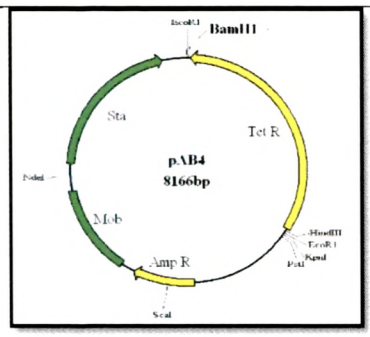
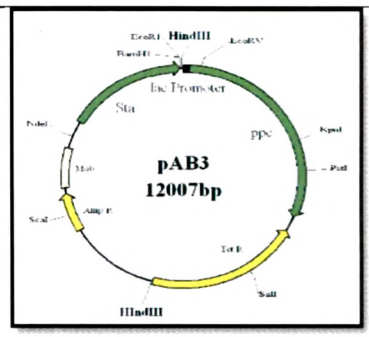
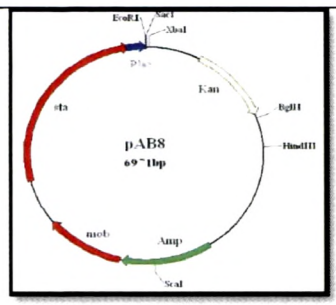
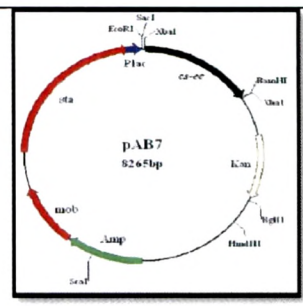
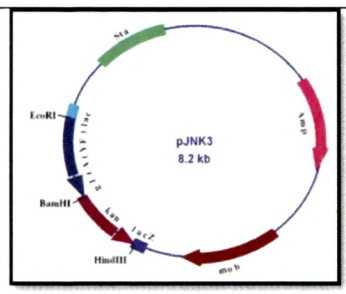
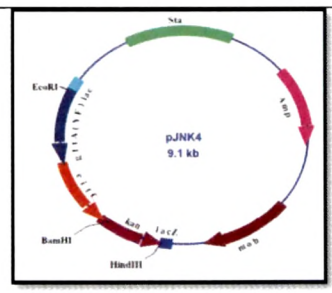
**Table 2.2: List of *Rhizobium* strains used in the present study.** *B. japonicum, M. loti* and *S. fredii* were used to incorporate genetic modifications and for further physiological studies.

| Bacterial Strains | Genotype | Reference |
|---|---|---|
| | ***Rhizobium* strains** | |
| *Bradyrhizobium japonicum* USDA110 | NC_004463.1 | NCBI |
| *Mesorhizobium loti* MAFF030669 | NC_002678.2 ←MAFF303099 | NCBI |
| *S. fredii* NGR 234 | NC_012587.1 | NCBI |
| *Bj* (pAB3) | *B. japonicum* with pAB3 plasmid; $Ap^r$, $Tc^r$ (*ppc*) | Chapter 3 |
| *Bj* (pAB4) | *B. japonicum* with pAB4 plasmid; $Ap^r$, $Tc^r$ (control vector) | Chapter 3 |
| *Bj* (pAB7) | *B. japonicum* with pAB7 plasmid; $Ap^r$, $Tc^r$ (*ppc*) | Chapter 4 |
| *Bj* (pAB8) | *B. japonicum* with pAB8 plasmid; $Ap^r$, $Tc^r$ (control vector) | Chapter 4 ,5 and 6 |
| *Bj* (pYF) | *B. japonicum* with pYF plasmid; $Ap^r$, $Km^r$ (*cs* NADH insensitive site direct mutation at 145 position Tyrosine is replaced by phenyl alinine) | Chapter 5 |
| *Bj* (pJNK4) | *B. japonicum* with pJNK4 plasmid; $Ap^r$, $Km^r$ (*cs* NADH insensitive and CitC transporter ) | Chapter 6 |
| *Ml* (pAB3) | *M. loti with* pAB3 *plasmid;* $Ap^r$, $Tc^r$ (*ppc*) | Chapter 3 |
| *Ml* (pAB4) | *M. loti* with pAB4 plasmid; $Ap^r$, $Tc^r$ | Chapter 3 |
| *Ml* (pAB7) | *M. loti* with pAB7 plasmid; $Ap^r$, $Km^r$ | Chapter 4 |
| *Ml* (pAB8) | *M. loti* with pAB8 plasmid; $Ap^r$, $Km^r$ | Chapter 4,5 and 6 |
| *Ml* (pYF) | *M. loti* with pYF plasmid; $Ap^r$, $Km^r$ | Chapter 5 |
| *Ml* (pJNK4) | *M. loti* with pJNK4 plasmid; $Ap^r$, $Km^r$ (*cs* NADH insensitive and CitC | Chapter 6 |

| | transporter | |
|---|---|---|
| *Bj* intYc | *Bradyrhizobium japonicum* USDA110 with the integrant | Chapter 7 |
| *Ml* intYc | *Mesorhizobium loti* MAFF030669 with the integrant | Chapter 7 |
| *Sf* intYc | *S. fredii* NGR 234 with the integrant | Chapter 7 and 8 |

**Table 2.3: List of plasmids used in the present study.** All the plasmids were stored as stocks at -20°C in the form of ethanol precipitates.

| Plasmids | Features | Reference |
|---|---|---|
| pUCPM18 | pUC18 derived Broad-Host-Range vector; $Ap^r$ | Hester et al., 2000 |
| pAB3 | pUCPM18 with *S. elongatus* PCC 6301 *ppc* geneunder *Plac* and *tc*$^r$ gene; $Ap^r$ /$Tc^r$ | Buch et al., 2008 |
| pAB4 | pUCPM18 with *tc*$^r$ gene; $Ap^r$, $Tc^r$ | Buch et al., 2008 |
| pAB7 | pUCPM18 with *E. coli cs* gene under *Plac* and *km*$^r$gene; $Ap^r$, $Km^r$ | Buch et al., 2008 |
| pAB8 | pUCPM18 with *km*$^r$ gene; $Ap^r$, $Km^r$ | Buch et al., 2008 |
| pJNK3 | pUCPM18 with NADH insensitive *cs* gene, $Ap^r$, $Km^r$ | Wagh, 2013 |
| pJNK4 | pUCPM18 with *citC* gene, $Ap^r$, $Km^r$ | Adhikary, 2012 |
| pGRG36 | Integration vector | Nancy et al., 2006 |
| pJIYC | Plasmid with yc operon | This study |

| | |
|---|---|
|  |  |
| **pUCPM18 with *tc*$^{r}$ gene; Ap$^{r}$, Tc$^{r}$ (Buch et al., 2008)** | **pUCPM18 with *S. elongatus* PCC 6301 *ppc* gene under P*lac* and *tc*$^{r}$ gene; Ap$^{r}$ /Tc$^{r}$ (Buch et al., 2008)** |
|  |  |
| **pUCPM18 plasmid containing Km$^{r}$ gene (Buch et al., 2008)** | **pUCPM18 plasmid containing wild type *E. coli* citrate synthase gene, Km$^{r}$ (Buch et al., 2008)** |
|  |  |
| **pUCPM18 with *E. coli* NADH insensitive *cs** gene under *Plac* , Ap$^{r}$ and km$^{r}$ gene (Wagh ,2013)** | **pUCPM18 with *E. coli cs** gene along with citrate transporter *citC* gene of *S.typhimorium* under *Plac* having Ap$^{r}$ and km$^{r}$ gene (Wagh ,2013)** |

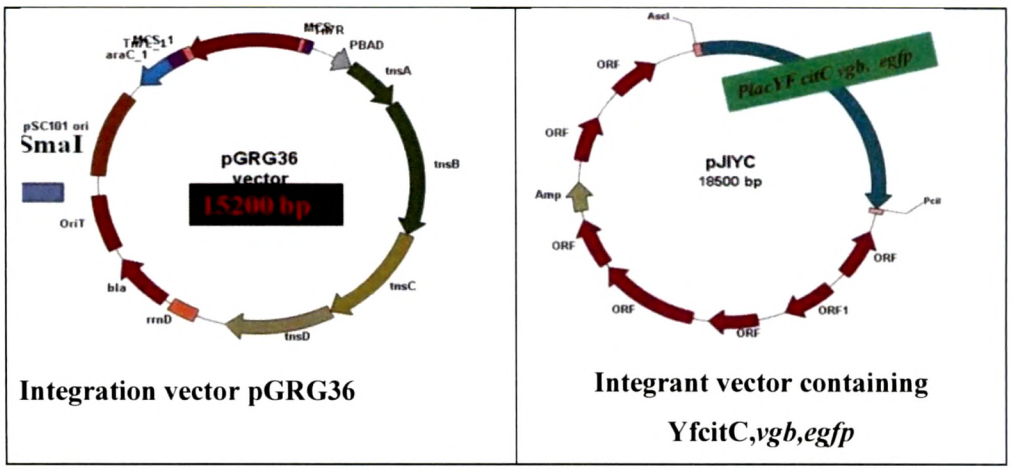



**Fig. 2.1: Restriction maps of the plasmids used in this study**

All plasmids are broad host- range vectors used for expression of heterologous overepression of genes under *lac* promoter in *Rhizobium* strains.

## 2.2: Media and Culture conditions

The *E. coli* strains and *Rhizobium* were cultured and maintained on Luria Agar (LA) and Yeast Extract Mannitol Agar (YEMA) respectively (Hi-Media Laboratories, India). *E. coli* cultures were grown at 37°C while all *Rhizobium* cultures were grown at 30°C. For growth in liquid medium, shaking was provided at the speed of 200 rpm. The plasmid transformants of both *E. coli* and *Rhizobium* were maintained using respective antibiotics at the final concentrations as mentioned in **Table 2.4** as and when applicable. Both *E. coli* and *Rhizobium* wild type strains and plasmid transformants grown in 3ml Luria broth (LB) containing appropriate antibiotics were used to prepare glycerol stocks which were stored at -20°C.

**Table 2.4: Recommended doses of antibiotics used in this study (Sambrook and Russell, 2001).** The antibiotic dozes were maintained same for both *E. coli* and pseudomonads. All the antibiotics were prepared in sterile distilled water or

recommended solvent at the stock concentrations of 1000x or 2000X (for antibiotics marked with *) and were used accordingly to have the desired final concentrations.

| Antibiotic | Rich medium | Minimal medium |
|---|---|---|
| Nalidix acid | 15 μg/ml | 3 μg/ml |
| Gentamycin* | 20μg/ml | 5 μg/ml |
| Tetracycline | 30μg/ml | 7.5μg/ml |
| Kanamycin | 50μg/ml | 12.5μg/ml |
| Streptomycin | 10μg/ml | 2.5μg/ml |
| Trimethoprim | 60μg/ml | 15μg/ml |
| Ampicillin* | 50μg/ml | 12.5μg/ml |
| Erythromycin | 100μg/ml | - |
| Chloramphenicol* | 20μg/ml | - |
| Spectinomycin | 50μg/ml | - |

The compositions of different minimal media used in this study are as described below. Antibiotic concentrations in all the following minimal media were reduced to 1/4th of that used in the above mentioned rich media (**Table 2.4**).

### 2.2.1: M9 minimal medium

Composition of M9 minimal broth was according to Sambrook and Russell (2001) including $Na_2HPO_4$ $7H_2O$, 34g/L; $KH_2PO_4$, 15g/L; $NH_4Cl$, 5g/L; NaCl, 2.5g/L; 2mM $MgSO_4$; 0.1mM $CaCl_2$ and micronutrient cocktail. The micronutrient cocktail was constituted of $FeSO_4.7H_2O$, 3.5 mg/L; $ZnSO_4.7H_2O$, 0.16 mg/L; $CuSO_4.5H_2O$, 0.08 mg/L; $H_3BO_3$, 0.5 mg/L; $CaCl_2.2H_2O$, 0.03 mg/L and $MnSO_4.4H_2O$, 0.4 mg/L. Carbon sources used were glucose, xylose, fructose and lactose as and when required. For solid media, 15g/L agar was added in addition to above constituents.

5X M9 salts, micronutrients (prepared at 1000X stock concentration) and carbon source (2M stock) were autoclaved separately. Fixed volumes of these were added aseptically into pre-autoclaved flasks containing distilled water to constitute the complete media with the desired final concentrations. Volume of water to be autoclaved per flask was calculated by subtracting the required volumes of each ingredient from the total volume of the media to be used.

### 2.2.2: Tris buffered medium

The media composition included Tris-Cl (pH=8.0), 100 mM; $NH_4Cl$, 10 mM; KCl 10 mM; $MgSO_4$, 2 mM; $CaCl_2$, 0.1 mM; micronutrient cocktail; Glucose, 100 mM and phosphate (P) sources (Sharma et al., 2005). 1mg/ml Senegal Rock phosphate (RP) or $KH_2PO_4$ were used as insoluble and soluble P sources respectively. Each ingredient was separately autoclaved at a particular stock concentration and a fixed volume of each was added to pre-autoclaved flasks containing sterile distilled water (prepared as in Section 2.2.3) to constitute complete media

### 2.2.3: Pikovskaya's (PVK) Agar

The media composition included Ammonium sulphate, 0.5g/L; Calcium phosphate, 5.0g/L; Dextrose, 10.0g/L; Ferrrous sulphate, 0.0001g/L; Magnesium sulphate, 0.1g/L; Manganese sulphate, 0.0001g/L; Potassium chloride, 0.2g/L; Yeast extract, 0.5g/L and agar, 15.0g/L. Dextrose was substituted by same amount of xylose and fructose as and when mentioned. The ready made media was obtained from Hi-Media Laboratories, India, and was used according to manufacturer's instructions.

### 2.2.4 Yeast Extract Mannitol Broth (YEMB)

Yeast Mannitol Agar with congo red was used to maintain *Rhizobium* species on plates, YEMA medium contains ingredients gms/ L. Yeast extract 1.000, Mannitol 10.000, Dipotassium phosphate 0.500, Magnesium sulphate 0.200, Sodium chloride 0.100, Congo red 0.025, Agar 20.000, Final pH ( at 25°C) 6.8±0.2 (Vincent, J.M. 1970).

### 2.2.5 Tryptone Yeast Extract Medium (TYE)

TY medium contained **0.5** % (w/v) Difco Bacto-Tryptone, 0.3 % (w/v) Difco Bacto-Yeast Extract and 7 mM-CaCl, Liquid PA medium contained 0.4 % (w/v) Difco Bacto-Peptone and **2** mM-MgSO,( Hirsch et al., 1980)

## 2.3: Morphological characterization and antibiotic sensitivity profile

Primary *Rhizobium* native strains were used for the antibiotic sensitivity profile was obtained for all native by checking the growth of these cultures by streaking on YEMA plates containing antibiotics. The same was also confirmed by inoculating single colonies of all the strains independently in 3ml TYE containing recommended dose of antibiotics (Sambrook and Russell, 2001) and checking the growth after overnight shaking at 30°C. The antibiotics used were erythromycin, ampicillin, kanamycin, spectinomycin, chloramphenicol, gentamycin, trimethoprim and tetracycline at the final concentrations as described in **Table 2.4.**

## 2.4: Molecular biology tools and techniques

### 2.4.1: Isolation of plasmid and genomic DNA

#### 2.4.1.1: Plasmid DNA isolation from *E. coli* and *B. japonicum* and *M. loti* transformants

The plasmid DNA from *E. coli* was isolated by the boiling lysis method using CTAB while that from *B. japonicum* and *M. loti* transformants was isolated using standard alkaline lysis method (Sambrook and Russell, 2001).

### 2.4.1.2: Genomic DNA isolation from *Rhizobium*

Fresh *Rhizobium* culture obtained by growing a single colony inoculated in 3ml TYE under shake conditions at 30°C was dispensed in 1.5 ml sterile centrifuge tubes, pelleted at 9, 200x *g* and washed twice with sterile normal saline. Following this, the cells were used for genomic DNA isolation performed using genomic DNA extraction kit (Cat.# FC46, Bangalore Genei, India) according to the manufacturer's instructions. The DNA was finally re-suspended in 40µl of sterile double distilled water.

### 2.4.2: Transformation of plasmid DNA

### 2.4.2.1: Transformation of plasmid DNA in *E. coli*

The transformation of plasmids in *E. coli* using $MgCl_2$-$CaCl_2$ method and antibiotic selection using tetracycline and kanamycin was carried out according to Sambrook and Russell (2001).

### 2.4.2.2: Transformation of plasmid DNA by Electroporation

Plasmid transformation in *Rhizobium* was done by electroporation as described by (Unge et al., 1998) *Rhizobium* cells grown till early stationary phase, Cells were chilled for 15 to 30 min on ice and then harvested by centrifugation at 9,000 rpm for 10 min at 4°C. in sorvall. . The cell pellet was washed four times with cold sterile deionized water final wash with 10% glycerol and resuspended 1 ml deionized water. The cell suspension was distributed in aliquots of 90 µl and mixed thoroughly with plasmid DNA (2 µg) and then kept on ice for 30 min. The cell-DNA mixture was loaded in a chilled electroporation cuvette with a 0.1-cm gap and was subjected to a single pulse of high voltage strength of up to 25 kV/cm .After the pulse was delivered, the cuvettes were kept on ice for 10 min .The cells were immediately transformed to 1 ml TYE broth and incubated for 24 h at 30°C. The cell suspension was diluted and plated on selective

medium (YEM agar plate with respective antibiotics/ml) and incubated at 30°C, for the selection of transformants.

### 2.4.3: Transfer of plasmid DNA by conjugation

The plasmids were transformed in *E. coli* S17.1, for mediating the conjugal transfer of the plasmids used (Section 2.4.2.1) and the resultant transformant strain was used as the donor strain. *E. coli* S17.1 harboring the plasmid and the recipient *Rhizobium* strain were separately grown in 3ml LB broth with respective antibiotics at 30°C under shake conditions for approximately 16h. The freshly grown cultures of recipient and the donor strains were aseptically mixed in 1:1 ratio (v/v) in a sterile centrifuge tube and the cells were centrifuged at 5000rpm for 5 minutes. The media supernatant was discarded to remove the antibiotics and the pellet was re-suspended in 0.2 ml of fresh sterile LB and the bacteria were allowed to mate at 30°C. After 16 h, the bacterial culture mix was centrifuged at 5000 rpm for 5 minutes and the resultant pellet was re-suspended in 0.2 ml of sterile normal saline. About 30 µl of this cell suspension was plated on YEMA containing the appropriate antibiotics for selection (antibiotic dose was as described in **Table 2.4**) to obtain the transconjugants. Conjugation mediated plasmid transfer was employed only for the native isolates of *Rhizobium* strains .

### 2.4.4: Agarose gel electrophoresis

The DNA samples were mixed with appropriate volume of 6X loading buffer (0.25% bromophenol blue, and 40% sucrose in water) and subjected to electrophoresis through 0.8% agarose (containing 1µg/ml ethidium bromide) gel in Tris-acetate-EDTA (TAE) buffer at 5v/cm for 0.5-2h. The DNA bands were visualized by fluorescence under the UV-light using UV transilluminator.

### 2.4.5: Restriction enzyme digestion analysis

0.5-1.0μg DNA sample was used for each restriction enzyme digestion. 1-3U of the restriction endonuclease (RE) was used with the appropriate 10X buffers supplied by the manufacturer in a final reaction volume of 10μl. The reaction mixture was incubated overnight at 37°C. The DNA fragments were visualized by ethidium bromide staining after electrophoresis on 0.8% agarose gels and were subsequently photographed. In case of double digestion, a compatible buffer for the two REs was essentially checked. If not available, digestion with one enzyme is performed followed by purification and subsequent digestion with the other enzyme, using respective buffers.

### 2.4.6: Gel elution and purification

The DNA fragments of desired sizes were recovered from the gel by cutting the agarose gel slab around the DNA band. The agarose piece was weighed in a sterile microcentrifuge tube and was solubilized in 2.5 volumes of 6M sodium iodide (NaI, freshly made) [e.g. for 200mg of agarose piece, 500μl of NaI was added]. Once completely dissolved, 15μl of silicon dioxide suspension (50% w/v, stored at 4°C) was added and was incubated at room temperature for 15-20 minutes. The DNA bound to silica was recovered by centrifuging at 9, 200x *g* for 2 minutes; the pellet was washed twice with 70% ethanol, dried, and finally re-suspended in 20-30μl sterile double distilled water. The microcentrifuge tube was incubated at 55°C for 10 minutes to allow complete dissociation of DNA from silica beads into the solution and then was subjected to centrifugation at 9, 200x *g* for 2 minutes. The resultant supernatant was gently recovered using sterile micropipette tip and was transferred to fresh sterile tube. The purification efficiency was checked by subjecting 2μl DNA solution to gel electrophoresis and visualizing the sharp DNA band of desired size. The purified DNA was used for ligation experiments only if >50ng/μl DNA was recovered after purification.

**2.4.7: Ligation**

The ligation reaction was usually done in 10µl volume containing the following constituents: Purified vector and insert DNA (volume varied depending on the respective Concentrations); 10X T4 DNA Ligase buffer, 1µl; T4 DNA ligase (MBI Fermentas), 0.5-1.0U and sterile double distilled water to make up the volume. The cohesive end ligation reaction was carried out at 16°C for 12-16h. The vector to insert molar ratio (molar concentrations calculated by the under mentioned formula) of 1:4 was maintained, with a total of 50-100ng of DNA in each ligation system.

$$\text{pmoles of DNA} = \frac{\text{Amount of DNA (µg) x 1, 515}}{\text{Size of the DNA fragment (no. of base pairs)}}$$

**2.4.8: Polymerase Chain Reaction (PCR)**

The PCR reaction set up was based on the guidelines given in Roche Laboratory Manual. The assay system and the temperature profile used are described in **Table 2.3**

**Table 2.5: PCR conditions used in the present study**

| Assay system used | | Temperature Profile | |
|---|---|---|---|
| Sterile DDW | 38 µl | Initial denaturation | 94°C- 5 min |
| dNTP(10mM with 2.5mM each) | 3 µl | Denaturation | 94°C- 30 sec |
| Reverse Primer 20pmoles | 1 µl | Annealing | Varies from 55-62°C for 30sec.* |
| Forward Primer 20pmoles | 1 µl | Elongation | 72°C for 45sec-2.5min* |
| Template DNA (100ng/µl) | 1 µl | | |
| Taq/20A PCR buffer (10X) | 5 µl | Final Elongation | 72°C- 10 min |
| Taq DNA/XT20 Polymerase (1 unit/µl)# | 1.0 µl | For plasmid | (30 cycles) |
| Total System | **50 µl** | For genomic DNA | (40 cycles) |

PCR amplifications were performed in Techne TC-312 thermal cycler. *Exact primer annealing temperature and primer extension time varied with primers (designed with respect to different templates) and has been specified in the text as and when applicable. Processivity of Taq polymerase is ~1000 bases per min. Taq DNA polymerase and its buffer, dNTPs and primers were obtained respectively from Bangalore Genei Pvt. Ltd., India, Sigma Chemicals Pvt. Ltd. and MWG Biotech. Pvt. Ltd, India, respectively and were used according to manufacturer's instructions. The theoretical validation of the primers with respect to absence of intermolecular and intramolecular complementarities to avoid primer-primer annealing and hairpin structures and the appropriate %G-C was carried out with the help of online primer designing software Primer 3. The sequence, length, and %G-C content of primers are subject to variation depending on the purpose of PCR and will be given as and when applicable in the

following chapters. The PCR products were analyzed on 1.0% agarose gel along with appropriate molecular weight markers (Section 2.4.4)

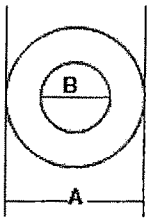
**2.4.9: Genome integration.**

pGRG36 plasmid used in the study for integration of genes in genome of *Rhizobium* strains, plasmid pGRG36 is temperature sensitive containing transposon miniTn*7*. Genes will get integrate at the *att* site of genome after electroporation of plasmid in *Rhizobium* strains, culture were kept at 42°C for 24 h for the integration (Nancy et al., 2006).

**2.5: P-solubilization phenotype**

P-solubilizing ability of the native as well as the transformant *Rhizobium* was tested on **(i)** Pikovskaya's (PVK) agar (Section 2.2.5; Pikovskaya, 1948) to monitor the ability to solubilize di-calcium phosphate and **(ii)** Tris buffered RP-Methyl red (TRP) agar plates which represent a much more stringent condition of screening for PSMs (Gyaneshwar et al, 1998). Liquid medium for RP solubilization is described in Section 2.2.2 with an addition of methyl Red as pH indicator dye and 1.5% agar for all the plate experiments. *Rhizobium* cell suspension for these experiments was prepared as described in Section 2.9.1 and 3µl of it was aseptically spotted on the above mentioned agar plates and was allowed to dry completely followed by incubation at 30°C for 5-7 days. P solubilization was determined by monitoring the zone of clearance on PVK agar and red zone on the TRP agar plates. Media acidification from pH=8.0 to pH<5 on TRP broth was used as an indicator for P-solubilization and organic acid secretion.

Phosphate solubilizing efficiency was calculated according to the formula:
PSI = (diameter of zone /diameter of colony) i.e. A/B.
The halo zones around the colonies were measured in mm.




**2.6: Physiological experiments**

The physiological experiments were carried out using various native and transformant *Rhizobium* which included growth, pH profile and enzyme assays.

**2.6.1: Inoculum preparation**

The inoculum for M9 and Tris minimal media containing free P was prepared by growing the ***Rhizobium*** cultures overnight at 30°C in 3ml LB broth. Inoculum for the buffered RP broth (TRP) was prepared by growing the ***Rhizobium*** cultures overnight at 30°C in 10 ml of M9 minimal medium. Cells were harvested aseptically, washed twice by normal saline, and finally re-suspended in 1ml normal saline under sterile conditions. Freshly prepared inoculum was used for all the experiments.

**2.6.2: Growth characteristics and pH profile**

Growth parameters and pH profile of the native as well as transformant ***Rhizobium*** were determined using three different media conditions including **(i)** TRP medium with RP as P source (Gyaneshwar et al, 1998). The media composition in this case was same as mentioned in Section 2.2.4; **(ii)** Tris buffered medium with $KH_2PO_4$ as P source: The media composition was same as mentioned in section 2.2.2 and **(iii)** M9 minimal medium (Section 2.2.1). 50mM glucose was used as the carbon source for all the experiments unless and until stated categorically. In 150ml conical flasks, 30ml of TRP broth containing RP was inoculated with cell suspensions to have 0.06-0.15 O.D600nm initially (0 hour O.D.). The batch culture studies were performed under aerobic conditions in Orbitek rotary shaker maintained at 30°C with agitation speed kept constant

at 200rpm. 1ml samples were aseptically harvested at regular intervals (varying with every set of batch culture depending on media conditions) and were subjected to various analytical techniques.

### 2.7: Estimation of Plant growth promoting factors

#### 2.7.1: Culture conditions for EPS production and quantification

Fresh overnight grown culture of *Rhizobium* strains transformants as well as native obtained by growing a single colony inoculated in 3ml LB under shake conditions at 30°C was dispensed in 1.5ml sterile centrifuge tubes, pelleted at 9, 200x *g* and washed thrice with sterile normal saline and used to for inoculation in 150ml flask containing 30ml M9 minimal medium supplemented with 50mM glucose as sole carbon source, cultures were grown at 30°C for 24h used to examine the EPS production. EPS was extracted using (EDTA) method described by (Tapia et al., 2009). Bacterial cells were centrifuged at 14,000 rpm for 20min supernatant was used for EPS extraction. EDTA (2% v/v) was added to supernatant for precipitation and kept at 40C for 2h, EPS obtained in each extraction were separated by centrifugation from solution and under 40C. EPS containing free cells solution was obtained by filtrated using nitrocellulose of 0.22 mm of pore size (Millipore). Filtered solution was used for EPS estimation. The phenol-sulphuric method was followed for total EPS estimation EPS (expressed as µg/ml) (Dubois et al., 1956) reaction mixture contained 1ml of EPS solution,1ml of aqueous phenol and 5ml of concentrated H2SO4 was mixed properly and kept for 15-20minutes, absorbance was measured at 490nm, the amount of EPS was determine against glucose as standard.

### 2.7.2: Bioflim assay

Samples were inoculated in test tube containing 3ml M9 minimal medium containing 50mM glucose concentration, Bacterial cultures were incubated under aerobic conditions in Orbitek rotary shaker maintained at 30°C for 24h with agitation speed kept constant at 200rpm. Growth medium was discarded after 24h from each test tube; to remove the unattached bacteria with test tubes were washed with Phosphate Buffer Saline (PBS). Biofilm were fixed with 2 ml of 99% methanol, tubes were left empty to dry. Crystal violet 2 ml of 1% was used for staining of adhered cells with 5 minutes incubation. Surplus stain was rinsed by distilled water for 5 times. Tubes were air dried and the dye attached to cells was dissolved in 1.5 ml of 33% glacial acetic acid. Biofilm formation was detected by simple tube method and estimated by measuring optical density (OD) at 570nm spectrophotometer assay described by Mathur et al., (2004).

### 2.7.3: Indole acetic acid (IAA) production and estimation

Overnight grown all cultures were pulled in sterile 1.5ml eppendorf in laminar, aspetic conditions were maintained , samples were washed 3 times by normal saline, these samples were used for inoculation of IAA production, cultures were inoculated in 150ml conical flask containing 30ml M9 minimal medium with L-tryptophan (0.1%) and one set without L-tryptophan with 50mM glucose as carbon source , flask were incubated at 300C orbital shaker 200rpm shaking for 24h, further samples were screened for IAA production, experiment was carried out in triplicates. Cell-free supernatant was obtained by centrifugation at 9000 × g for 20 minutes; supernatant was used for IAA extraction method described by (Sinha and Basu, 1981). To the 10 ml of supernatant, 2 ml of salkowski's reagent (1 ml of 0.5 M FeCl3 + 50 ml of 35% perchloric acid) was added and incubated for 30 mintues under darkmess. Pink colour formed was determined colorimetrically at 540 nm as IAA production.

## 2.8: Analytical techniques

The cell density determinations were done at 600nm as monitored spectrophotometrically. Change in absorbance was considered as the measure of growth and drop in pH of the media was taken as the measure of acid production. In all the cases, the observations were continued till the media pH reduced to less than 5. 1ml aliquots withdrawn aseptically at regular time intervals were immediately frozen at - 20°C until further used for biochemical estimations. The stored samples were centrifuged at 9, 200x *g* for 1 min at 4°C and the culture supernatants derived were used to estimate residual glucose and organic acid analysis using HPLC. For HPLC analysis, the culture supernatant was passed through 0.2μm nylon membranes (MDI advanced microdevices, India) and the secreted metabolites were quantified using RP-18 column. The column was operated at room temperature using mobile phase of 0.01M $H_2SO_4$ at a flow rate of 1.0 ml $min^{-1}$ and the column effluents were monitored using a UV detector at 210 nm. For citric acid estimation the same column was operated at room temperature using mobile phase of 20mM $Na_2HPO_4$ with 2.5% acetonitrile at a flow rate of 1.0 ml $min^{-1}$. Standards of organic acids were prepared in double distilled water, filtered using 0.2 μm membranes and were subjected to chromatography for determining the individual retention time. Measurements of area under peak with an external standard were used for quantification. The glucose concentration in the medium was estimated using enzymatic kit (Reckon Diagnostics, India).

The physiological parameters like specific growth rate, specific total glucose utilization rate and biomass yield (as described by Chao and Liao, 1993) and the organic acid yield were calculated as described below.

**(i) Specific growth rate (h-1):**

$$k = \frac{(\text{Log10Nt1} - \text{Log10Nt2}) \times 3.3}{(t1 - t2)\ (h)} \quad \text{where,}$$

N1 and N2 are the number of cells at time t1 and t2 respectively and (t1-t2) is the corresponding time interval in hours. 3.3 is the factor derived from the formula- number of generations (n) = (Log10N-Log10N0)/Log102. The number of cells was calculated from O.D.600nm using the correlation 1 O.D.600nm = 1.5x109 cell/ml (Koch et al., 2001)

**(ii) Specific total glucose utilization rate (QGlc):**

$$QGlc = \frac{\Delta\text{Glucose}\ (t1 - t2)\ (g/L)}{\Delta\text{dcw}\ (t1 - t2)\ (g/L) \times \text{Time interval}\ (t1 - t2)\ (h)} \quad \text{where,}$$

ΔGlucose (t1-t2) is the amount of glucose consumed over the time interval t1-t2; Δdcw (t1-t2) is the difference in the dry cell weight (dcw) of the cells over the time interval t1-t2. QGlc is expressed as g glucose utilized/g dcw/h). Dry cell weight was calculated using the correlation 1 O.D.600nm = 0.382mg/ml (Bugg et al., 2000).

**(iii) Biomass yield**

$$Ydcw/Glc = \frac{\Delta\text{dcw}\ (t1 - t2)\ (g/L)}{\Delta\text{Glucose}\ (t1 - t2)\ (g/L) \times \text{Time interval}\ (t1 - t2)\ (h)} \quad \text{where,}$$

All the parameters were as described for Specific glucose utilization rate. Ydcw/Glc is expressed as g of dry cell weight produced/ g glucose utilized/h.

**(iv) Organic acid yield**

$$\text{Organic acid yield} = \frac{\text{Amount of organic acid produced (g/L)}}{\text{Total glucose utilized (g/L) x dcw (g/L)}}$$ where,

The amount of total glucose utilized was obtained by deducting the value of residual glucose concentration from the initial glucose concentration supplied in the medium. The difference between the total glucose utilized and gluconic acid produced was considered as glucose consumed. Hence, the total glucose utilized and not glucose consumed was taken into account for calculating specific glucose utilization rate. The statistical analysis of all the parameters was done using Graph Pad Prism (version 3.0) software and microsoft Excel.

**Dry cell mass (dcw)** = O.D600nm x 0.382, where 0.382 is the factor correlating O.D600 was with dry cell weight (Bugg et al., 2000)

## 2.9: Enzyme assays

### 2.9.1: Preparation of cells and cell free extracts

Glucose grown cells under above mentioned minimal media conditions were harvested in appropriate growth phase from 30ml of cell culture by centrifugation 9,200x *g* for 2 minutes at 4°C. Unlike citrate synthase (CS), isocitrate lyase (ICL) and isocitrate dehydrogenase (ICDH) which were assayed in the stationary phase, all the enzyme were assayed from mid-late log phase cell cultures. The preparation of cell free extracts for PPC, CS, PYC, G-6-PDH, ICDH and ICL assays was carried out according to Kodaki et al (1985) with an addition of 5 mM $MgCl_2$ and 1 mM EDTA for PYC assay. The cell pellet was washed once with 80mM phosphate buffer (pH=7.5) followed by resuspension in same buffer containing 20% glycerol and 1 mM DTT. The cells were then subjected to lysis by sonicating for maximum 1-1.5 minute in an ice bath, followed by centrifugation at 9,200x *g* at 4°C for 30 minutes to remove the cell debris. The supernatant was then used as cell-free extract for the enzyme assays. The whole cell preparation for GDH

assay was done by washing the harvested cells (mid-late log phase cultures) thrice with normal saline to remove the residual glucose of the medium and resuspending in 0.01M hosphate buffer (pH 6.0) with 5 mM $MgCl_2$.

### 2.9.2: Enzyme Assay Protocols

#### 2.9.2.1: PPC assay

PPC (EC 4.1.1.31) activity was estimated spectrophotometrically by monitoring the oxidation of NADH in a coupled assay with malate dehydrogenase (MDH) as described by Kodaki et al (1985) with modifications as follows. The coupled assay was divided in two steps. The assay mixture for first step of the reaction contained following ingredients in total volume of 0.9ml: Tris-$H_2SO_4$ (pH 8.0), 100 mM; potassium PEP, 2 mM; $MgSO_4$, 10 mM; $KHCO_3$, 10 mM and the enzyme solution (cell lysate).

After 20 minutes incubation at 30°C, the reaction was terminated using 25% TCA followed by 15 minutes incubation on ice bath. The supernatant recovered after centrifuging the resultant reaction mix at 9,200x *g* for 30 minutes at 4°C, was brought to pH 8.0 by using 7µl of 10N NaOH which was then used as the source of OAA in second step of the reaction for which the assay system contained 950µl of reaction mix from first step of reaction, 5units of MDH and 0.12mM NADH. The assay system volume was adjusted to 1ml using distilled water. The rate of oxidation of NADH recorded at 340nm was used to indicate concentration of OAA formed. Molar absorbance of NADH was taken as 6.22mM-1cm-1 at pH 8.0.

*The absence of glycerol in the sonication buffer led to complete loss of PPC activity* (Kodaki et al., 1985).

#### 2.9.2.2: PYC assay

PYC (EC 6.4.1.1) activity was estimated by monitoring NADH oxidation in a coupled assay with MDH (Taylor et al., 1972) with modifications as follows. The assay mixture for first step of the reaction contained following ingredients in total volume of 0.9 ml: Tris-HCl (pH 8.0), 100 mM; Na-pyruvate, 5 mM; ATP, 5 mM; $MgCl_2$, 5 mM; $NaHCO_3$, 50 mM and the enzyme solution (cell lysate). Remaining steps and specific activity determination were same as in case of PPC activity.

**2.9.2.3: G-6-PDH assay**

G-6-PDH (EC 1.1.1.49) activity was determined spectrophotometrically at room temperature by following the reduction of NADP at 340nm as a function of time (Eisenberg and Dobrogosz, 1967). Molar absorbance of NADP was taken as 6.22 $mM^{-1}cm^{-1}$ at pH 8.0. The reaction mixture of 1ml included: Tris-Cl (pH 8.2), 200 mM; glucose-6-phosphate, 3.3 mM; $MgCl_2$, 10 mM; NADP, 0.1mM and cell extract.

**2.9.2.4: GDH assay**

GDH (D-glucose phenazine methosulphate oxidoreductase, (EC 1.1.5.2) was determined spectrophotometrically by following the coupled reduction of 2,6-dichlorophenolindophenol (DCIP) at 600nm (Quay et al., 1972). Molar absorbance of DCIP was taken as 15.1 mM-1 cm-1 at pH 8.75. The reaction mixture included: Tris-Cl buffer (pH 8.75), 16.66 mM; D-glucose, 66 mM; DCIP, sodium salt, 0.05 mM; phenazine methosulfate, 0.66 mM; sodium azide, 4 mM; whole cells, and distilled water to 3.0ml.

**2.9.2.5: ICL assay**

ICL (4.1.3.1) activity was measured by a modified method of Dixon and Kornberg (1959). ICL catalyzes the hydrolysis of isocitrate into glyoxylate and succinate. The glyoxylate formed in the presence of phenylhydrazine was measured as glyoxylic acid phenylhydrazone at 324nm. The reaction mixture of 1ml consisted of following ingredients: potassium phosphate buffer (pH=7.0), 100 mM; $MgCl_2$, 6 mM; cysteine HCl, 12 mM; phenylhydrazine HCl, 4 mM; isoctrate; 8 mM and cell extract. Molar absorbance coefficient of phenylhydrazine was taken as 17.4 $mM^{-1}cm^{-1}$. The rate of increase in absorbance in the linear range was used to calculate ICL activity.

**2.9.2.6: ICDH assay**

ICDH (1.1.1.42) activity was measured by following NADPH formation at 340nm (Garnak and Reeves, 1979). The reaction mixture contained the following in 1ml: Tris-HCl (pH 7.5), 150 mM; $MnCl_2$, 0.25 mM; NADP, 0.65 mM, isocitrate, 2.5 mM and cell extract. Molar absorbance coefficient of NADP was taken as 6.22 $mM^{-1}cm^{-1}$.

**2.9.2.7: CS assay**

CS (4.1.3.7) activity was estimated by following the absorbance of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) at 412nm which would change due to its reaction with sulfhydryl group of CoA (Serre, 1969). The assay mixture per cuvette contained following ingredients in 1.0 ml: Tris-HCl (pH=8.0), 93 mM; acetyl CoA, 0.16 mM; OAA, 0.2 mM; DTNB, 0.1 mM and cell lysate. The reaction was started by addition of OAA. Molar absorbance coefficient was taken as 13.6 $mM^{-1}cm^{-1}$ at 412nm. The rate of increase in absorbance was used to calculate CS activity.

All the enzyme activities were determined at 30°C and were expressed per mg total protein. Total protein concentration of the crude extract as well as whole cell suspensions was measured by modified Lowry's method (Peterson, 1979) using bovine serum albumin as standard. Corrections were made for Tris buffer. Enzyme activities were calculated using following formula:

$$\text{Specific enzyme activity (U)} = \frac{\Delta A_y \text{ nm/min}}{\epsilon \times \text{enzyme (sample) aliquot (ml)} \times \text{Total protein (mg/ml)}}$$ where,

$\Delta A_y$ nm is the difference in the absorbance at any given wavelengths (y nm) and $\epsilon$ is the millimolar extinction coefficient at *y* nm.

One unit of enzyme activity was defined as the amount of protein required to convert 1 nmole of substrate per minute unless stated in the figure legend.

**2.10: Inoculation of mung beans (*Vigna radiata*)**

Mung bean seeds were surface sterilized by treating with 0.1% (w/v) mercuric chloride for ~3-5 minutes followed by 5 rounds of thorough washing with 100ml freshly sterilized distilled water (Ramakrishna et al., 1991). These seeds were placed in autoclaved petri-dishes containing moist filter paper and were allowed to germinate in dark for 24h at room temperature. *Rhizobium* was inoculated in 10ml sterile TYE broth

and was allowed to grow at 30°C with overnight shaking. Fresh culture was harvested by centrifuging at 9, 200x *g* for 2 minutes; the pellet was aseptically washed twice with sterile normal saline and finally was re-suspended in 4 ml of normal saline (Ramos et al., 2000). Healthy germinated seeds were incubated with this cell suspension in a sterile petri-dish under aseptic conditions for 10-15 minutes. Root tips of hence obtained *Rhizobium* coated mung bean seeds were planted in pots; soil used in experiment was unsterilized. Plants were allowed to grow till 45 days in green house.

## 2.11 Pot experiments- Interaction with mung beans

### 2.11.1 Preparation of inoculum

A loopful of bacterial strain was inoculated into a 50 ml aliquot of TYE broth and incubated at 30°C for 24 h in an incubator shaker.

### 2.11.2Growth Analysis

### 2.11.2.1 Growth Analysis: Above ground parts

**Plant height:**

Measurements of plant height were taken at the crop maturity in three replicates of five plants each. Plant height was measured from the soil line to shoot tip. Plant height was measured by placing the plant on a centimeter scale. Plant population was uniform at the time of maturity of crop.

**Leaf area**:

The area of the leaf was taken by pressing the leaf on millimeter graph paper and tracing the exact outline. The area was measured by weighing the graph cuttings of the leaf (Shine et al., 2011). The calibration curve was prepared by weighing a 0–100 $cm^2$ area of graph paper.

**Dry matter production**

At maturity fifteen plants (five plants from each replicate) were randomly selected and the data on component part dry weights (leaf, root and shoot) were recorded. Leaf,

stem and root were dried at 60 °C for 72 hours. Pods were separated and the total numbers of branches were recorded. Dry weights of seed components were recorded after drying at 35–40 °C for 10 days.

**Physical and chemical properties of experimental soil used for *Rhizobium* strains integrants**

| pH | P Kg/hac$^{-1}$ | N Kg/hac$^{-1}$ | K Kg/hac$^{-1}$ |
|---|---|---|---|
| 8 | 12.9 | 158.7 | 338.7 |

**2.11.2.2 Growth Analysis: Below ground parts**

**Root length:**

The measurements of root length were done in plants at 20 and 45 DAI. Roots were taken out carefully, washed, and measured against a cm scale.

**Root fresh and dry weight**

Plant roots with nodules at each sampling were washed and dried on filter paper and weighed for the fresh weight. For dry weight roots were dried at 60oC for 72 hours and weighed.

**Number of root nodules/plant**

Nodules on each root were counted carefully and recorded per plant.

**Nodule fresh weight**

Nodules were taken out at each sampling, washed, and dried on filter paper. Weight of 10 nodules was recorded in g.

**Acetylene Reduction Assay (ARA): Measuring Nitrogenase Activity** – ARA assay was performed on GC at SICART method described by Castle 2010.

### 2.11.3 Antioxidant Enzymes / ROS scavenging enzyme activity:

#### 2.11.3.1 Superoxide Dismutase (SOD)

Two hundred mg of leaves were homogenized in a pre-chilled mortar and pestle under ice cold condition using 3.0 ml of extraction buffer, containing 50 mM sodium phosphate buffer (pH 7.4), 1 mM EDTA and 1% (W/V) polyvinylpyrolidone (PVP). The homogenates were centrifuged at 10,000 rpm for 20 minutes and the supernatants used for the assay (Costa et al., 2002).

Total SOD (EC 1.15.1.1) activity was measured spectrophotometrically based on inhibition in the photochemical reduction of nitroblue tetrazoilum (NBT). The 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA and 0.1 ml enzyme extract, riboflavin was added last (Van Rossun et al., 1997).After addition of all these components and mixing, test tubes were placed on stand 30 cm below a light source consisting of four 15-w fluorescent lamps. The photochemical inhibition was allowed to happen for 10 minutes and stopped by switching off the light source. The photoreduction in NBT was measured as increase in absorbance at 560 nm. Blanks and controls were run the same way but without illumination and enzyme, respectively. One unit of SOD was defined as the quantity of enzyme required to inhibit the reduction of NBT by 50% in a reaction mixture. Enzyme unit of SOD was calculated according to formula given by Constantine and Stanley (1977).

$$\text{SOD unit} = \frac{\text{Control O.D. (without enzyme)}}{\text{Sample O.D.}} - 1 \times \frac{1}{\text{(g) conc. Enzyme}}$$

$$\text{SOD U/g protein} = \frac{\text{SOD unit}}{\text{protein mg/g}}$$

### 2.11.3.2 Catalase

Two hundred mg of acetone powder homogenized with a pre-chilled mortar and pestle under ice cold condition in 2.0 ml of extraction buffer, containing 0.1 M sodium phosphate buffer (pH 7.2) with the addition of 1 mM EDTA and 1% (w/v) polyvinylpyrolidone (PVP) and a pinch of activated charcoal. The homogenates were centrifuged at 10,000 rpm for 20 minutes and the supernatants were used for the assay (Mahatma et al., 2011).

Total catalase (EC 1.11.1.6) activity was determined in the supernatants by measuring the decrease in absorption at 240 nm as $H_2O_2$ ($\varepsilon$ = 39.4 mM-1 cm-1) got consumed according to the method of Aebi (1984) and enzyme activity expressed as mmol $H_2O_2$ oxidized $min^{-1}$ $g^{-1}$.protein. The 3 ml assay mixture contained 50 mM sodium phosphate buffer (pH 7.0), 30mM $H_2O_2$ and 50 µl enzyme extract. Enzyme unit of CAT was defined as:

$$\text{Mmol/min/g protein} = \frac{\text{O.D. } \Delta}{\varepsilon \times \text{protein mg/g} \times \text{Enzyme conc. (g)}}$$

Where, $\varepsilon$ = Extinction coefficient, Enzyme conc. (g) = Amount of enzyme in 3 ml reaction mixture

### 2.11.3.3 Guaiacol Peroxidase (POX)

Two hundred mg of leaves were homogenized in a pre-chilled mortar and pestle under ice cold condition in 2.0 ml of extraction buffer, containing 0.1M sodium phosphate buffer (pH 7.2) with the addition of 1 mM β-mercaptoethanol and 1% (w/v) polyvinylpyrolidone (PVP). The homogenates were centrifuged at 10,000 rpm for 20 minutes and the supernatants used for the assay.

POX (EC 1.11.1.7) activity was determined in the supernatants of centrifuged homogenates by measuring the increase in absorption at 470 nm due to the formation of tetraguaiacol ($\epsilon$=26.6$mM^{-1}cm^{-1}$) in a reaction mixture containing 50 mM sodium

phosphate buffer pH 7.0, 0.1 mM EDTA, 0.05 ml enzyme extract, and 10 mM $H_2O_2$ (Costa et al.,2002).

### 2.11.3.4 Ascorbate peroxidase (APX)

Maize leaf samples were crushed with chilled acetone in pre-chilled mortar-pestle. To obtain fine acetone powder the homogenates were filtered and stored immediately at - 20o C. Hundred mg of acetone powder was then homogenized in a pre-chilled mortar and pestle under ice cold condition using 2.0 ml of extraction buffer, containing 0.1 M sodium phosphate buffer (pH 7.2), and 1% (w/v) polyvinylpyrolidone (PVP). The homogenates were centrifuged at 10,000 rpm for 20 minutes and the supernatants used for the assay (Mahatma et al., 2011).

APX (EC 1.11.1.11) activity was measured immediately in fresh crude extracts and assayed by procedure described by Nakano and Asada (1981). Three ml of the reaction mixture contained 50 mM sodium phosphate buffer pH 7.0, 0.1 mM $H_2O_2$, 0.5 mM ascorbic acid, 0.1 mM EDTA and 0.1 ml enzyme extract. The hydrogen peroxide dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$). The enzyme unit of APX was defined similar to that of CAT activity as described.

### 2.11.4: Acetylene Reduction Assay (ARA): Measuring Nitrogenase Activity

ARA assay was performed from root nodules on GC at SICART method described by Castle 2010.

### 2.11.5 Chlorophyll Content

Total chlorophyll content of fresh leaves was estimated according to method described by Khaleghi et al., 2012.

### 2.11.6 Estimation of Water Soluble Protein Content

Protein concentration of each enzyme extract was estimated by method of Lowry et al., (1951).

**(a) Reagents for Lowry's Method**

(i) Solution A: 2% Na2CO3 in 0.1 N NaOH

(ii) Solution B: (a) 1% CuSO4.5H2O solution

(b) 2% sodium potassium tartarate solution

Working solution of B: Prepared fresh before use by mixing equal volume of solution B (a) and B (b).

(iii) Solution C: Prepared fresh before use by mixing 50 ml of solution A and 1 ml of working solution of B.

(iv) Solution D: Folin and Ciocalteu reagent (1N).

**Procedure (Folin Lowry's Method)**

Enzyme extracts (25 µl) were taken in test tube and volume was made up to 1 ml with millipore water. A tube with 1 ml of water served as blank. Five ml of solution C was mixed by vortexing and kept for 10 min. Then 0.5 ml of solution D (Folin and Ciocalteau reagent) was added and vortexed. The tubes were allowed to stand at room temperature for 30 min. Absorbance was read at 660 nm. A standard curve was prepared using bovine serum albumin (BSA) in the concentration range of 10-80 µg.