

Chapter 2

Materials and Methods

2: Materials and Methods

2.1 Bacterial strains / Plasmids

All the plasmids use in the present study and their restriction maps are given in **Table 2.1**, **Fig. 2.1** and **Fig 2.2**. The wild type and genetically modified *E. coli* and *Herbaspirillum seropedicae* Z67 strains are listed in **Table 2.1** *E. coli* DH10B was used for all the standard molecular biology experiments wherever required. *Herbaspirillum seropedicae* Z67 strain used in all this study is generous gift from (Dr E.K. James) The *gltA* (citrate synthase gene) mutant of *E. coli* was obtained from *E. coli* Genetic Stock Center (CGSC), Yale University, U.S.A. The NADH insensitive *cs* mutant plasmid was a generous gift from Prof Harry Duckworth, University of Manitoba, Canada. The *pqqE* gene was gifted by Goldstine Department of Biology, California State University at Los Angeles, State University Drive, Los Angeles, California. *pqq* gene cluster of *Acinetobacter calcoaceticus* was gifted by (Dr. Goosen, Molecular Genetics, University of Leiden, Netherland) and *pqq* gene cluster of *Pseudomonas fluorescens* B16 was generous gift by (Dr. Ingyu Hwanag, Department of Biochemistry, Seoul University, Korea).

Table 2.1: List of plasmids used in the present study.

Plasmid/Strains	Characteristics	Source orReference
<i>E. coli</i> DH10B	Used for maintaining plasmids. <i>Sir⁺F⁺ endA1 recA1 galE15 galK16 nupGrpSL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara.leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) Δ</i>	(Invitrogen)
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap ^r	Hester et al., 2000
pAB8	pUCPM18 with km ^r gene; Ampr, Km ^r	Buch et al., 2009
pAB7	<i>gluA</i> gene of <i>E. coli</i> in pAB8, Apr/Km ^r	Buch et al., 2009
pY145F	NADH insensitive <i>cs*</i> gene	Duckworth, H., 2003
<i>citC</i>	Na ⁺ dependent citrate transporter of <i>Salmonella typhimorium</i>	Chapter 3
pJNK3	pUCPM18 with <i>E. coli</i> NADH insensitive <i>cs*</i> gene under <i>Plac</i> and km ^r gene; Ap ^r , Km ^r	Chapter 3
pJNK4	pUCPM18 with <i>E. coli</i> NADH insensitive <i>cs*</i> gene citrate transporter <i>citC</i> of <i>Salmonella typhimorium</i> under <i>Plac</i> and km ^r gene; Ap ^r , Km ^r	Chapter 3
<i>Hs</i> Z67	Nitrogen fixer, Natural isolate from gramenae plant; Nal ^r	Baldani et al., 1986a
<i>II_s</i> (pAB8)	<i>H. seropedicae</i> Z67 pUCPM18 with <i>npII</i> gene, Ap ^r /Km ^r	Chapter 3

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to enhance nitrogen fixation ability.

<i>Hs</i> (pAB7)	<i>H. seropedicae</i> Z67 Nal ^r with pAB7, Km ^r	Chapter 3
<i>Hs</i> (pJNK3)	<i>H. seropedicae</i> Z67 Nal ^r with pJNK3, Km ^r	Chapter 3
<i>Hs</i> (pJNK4)	<i>H. seropedicae</i> Z67 Nal ^r with pJNK4, Km ^r	Chapter 3
pBBR1MCS-2	Broad-Host-Range vector; Km ^r	Kovach et al., 1995
pMCG898	Containing <i>pqqE</i> of <i>Erwinia herbicola</i>	Goldstein and Liu, 1987
pJNK1	pBBR1MCS-2 Kmr with 1.8 kb <i>E. herbicola pqqE</i> gene	Chapter 4
pSS2	25Kb plasmid contains 5.1-kb of <i>pqq</i> gene cluster of <i>Acinetobacter calcoaceticus</i>	Goosen et al., 1989
pOK53	35Kb plasmid contains 13.4-kb <i>pqq</i> gene cluster of <i>Pseudomonas fluorescens</i> B16	Choi et al., 2008
<i>E. coli</i> DH10B	<i>Str r F- endA1 recA1 galE15 galK16 mupG rpsL AlacX74 φ80lacZ ΔM15 araD139 Δ(ara, leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ-</i>	Invitrogen
<i>Il</i> s Z67	Nitrogen fixing rice endophyte, Nal ^r	Baldani et al., 1986a
<i>Hs</i> (pBBR1MCS2)	<i>H. seropedicae</i> Z67 with (pBBR1MCS2) Km ^r	Chapter 4
<i>Hs</i> (pJNK1)	<i>H. seropedicae</i> Z67 with pJNK1 Km ^r	Chapter 4

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Hs (pSS2)	<i>H. seropedicae</i> Z67 with pSS2 Tet ^r	Chapter 4
Hs (pOK53)	<i>H. seropedicae</i> Z67 with pOK53Tet ^r	Chapter 4
<i>Enterobacter asburiae</i> PSI3	Amp ^r secretes gluconic acid	Gyaneshwar et al., 1999
<i>Pseudomonas aeruginosa</i> P4	Secretes gluconic acid	Buch et al., 2008
pSS160	pUC18 plasmid contains 5.1-kb of <i>pqq</i> gene cluster of <i>Acinetobacter calcoaceticus</i>	Goosen et al., 1989
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap ^r and Gen ^r	Hester et al., 2000
pCNK12	pJET2.1 with <i>gad</i> operon	Chanchal et al., 2013
pJNK5	<i>Pqq</i> gene cluster of <i>Acinetobacter calcoaceticus</i> in pUCPM18, Apr and Gen ^r	Chapter 5
pJNK6	<i>gad</i> operon of <i>Pseudomonas putida</i> KT2440 in pJNK5, Apr and Gen ^r	Chapter 5
Hs (pUCPM18)	<i>H. seropedicae</i> Z67 with pUCPM18 Broad-Host-Range vector; Ap ^r and Gen ^r	Chapter 5
Hs (pJNK5)	<i>H. seropedicae</i> Z67 with pJNK5, Ap ^r and Gen ^r	Chapter 5
Hs (pJNK6)	<i>H. seropedicae</i> Z67 with pJNK6, Ap ^r and Gen ^r	Chapter 5

Genetic modification of *Herbaspirillum seropedicae* Z67
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to enhance nitrogen fixation ability.

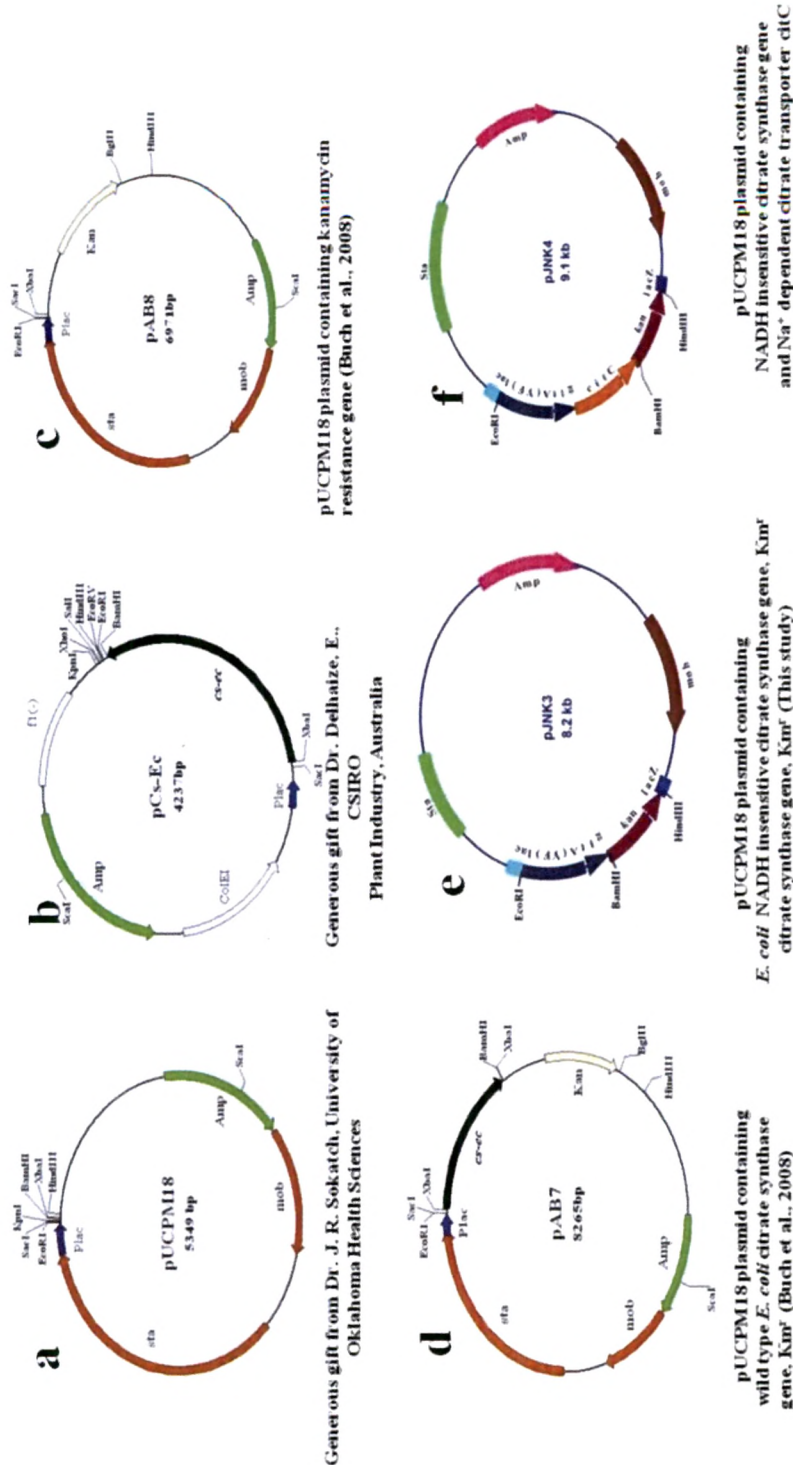


Fig. 2.1: Restriction maps of the plasmids used in this study. All plasmids are broad host-range vectors used for expression of heterologous overexpression of genes under *lac* promoter in *Herbaspirillum seropedicae* Z67

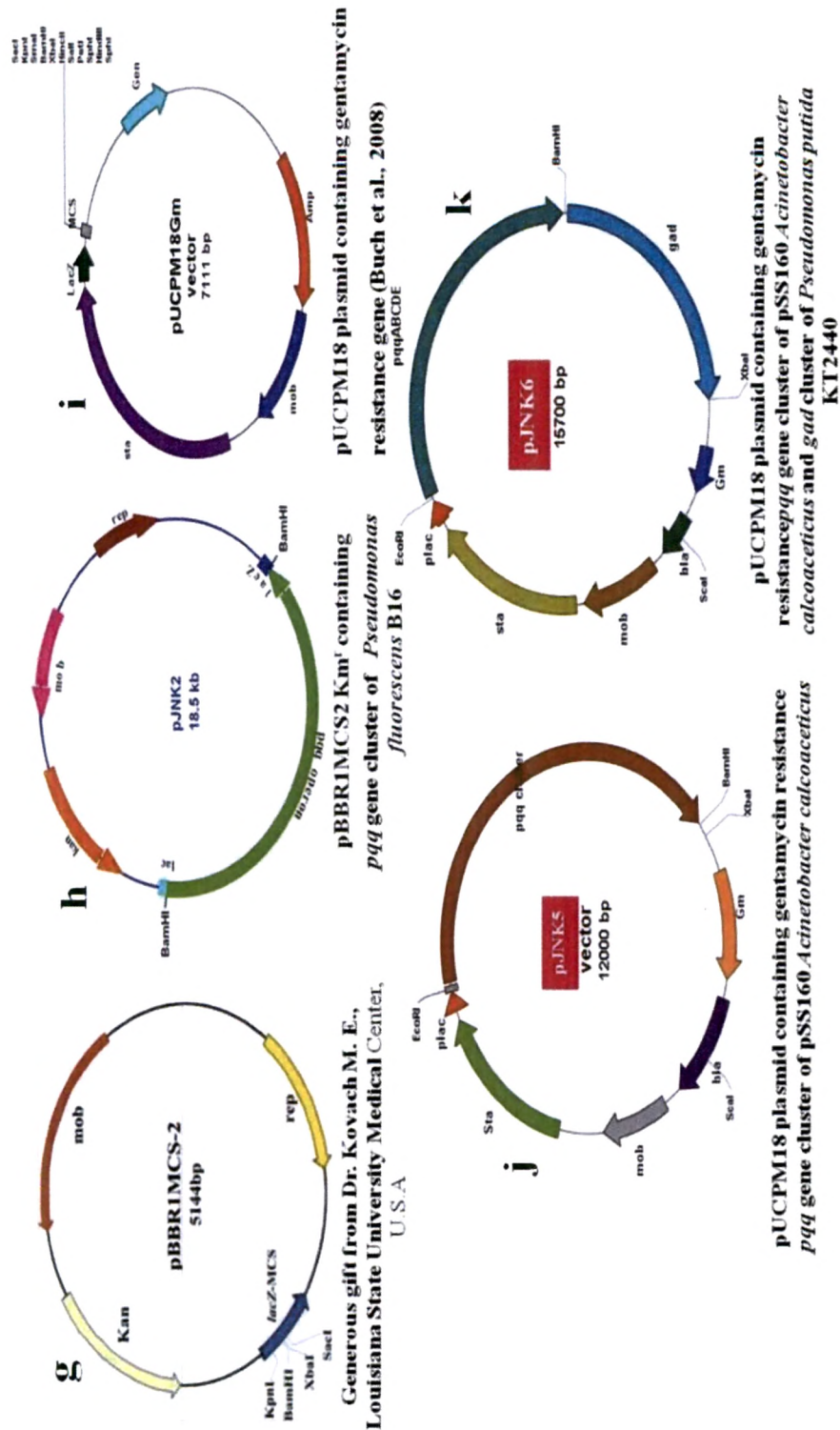


Fig. 2.2: Restriction maps of the plasmids used in this study. All plasmids are broad host-range vectors used for expression of heterologous overexpression of genes under *lac* promoter in *Herbaspirillum seropedicae* Z67

2.1.1: pUCPM18 plasmid is derived from a pUC-derivative, pUCP18 plasmid which is stably maintained in both *E. coli* and *Pseudomonas* species (Schweizer, 1991). pUCPM18 was developed from pUCP18 (GenBank accession number: U07164) by incorporating a 750bp mob fragment from pLAFRI (broad host range vector used for genetic analysis of gram-negative bacteria; Vanbleu et al., 2004), in order to enable convenient mobilization within *Pseudomonas* species (Hester et al, 2000). It replicates in *E. coli* using ColE1 origin of replication (*ori*) while in *Pseudomonas* it replicates owing to a pRO1614 derived DNA fragment encoding a putative *ori* and a replication-controlling protein (West et al., 1994).

2.1.2: pBBR1MCS-2 (GenBank accession number: U23751) plasmid conferring kanamycin resistance is originally derived from pBBR1 plasmid of *Bordetella bronchiseptica* (Kovach et al., 1995). This plasmid facilitates cloning due to availability of unique restriction sites and direct selection of recombinant plasmids based on blue white selection strategy. pBBR1MCS based vectors are mobilizable when the RK2 transfer functions are provided in *trans*, are compatible with IncP, IncQ and IncW group plasmids, as well as with ColE1- and P15a-based replicons, can stably replicate in a variety of hosts including *E. coli*, *P. fluorescens*, *P. putida*, *Rhizobium meliloti* and *R. leguminosarum* bv. *Viciae* and are stably retained *in vivo* for more than 4 weeks in absence of antibiotic selection (Kovach et al., 1995).

2.2: Media and Culture conditions

The *E. coli* strains and pseudomonads were cultured and maintained on Luria Agar (LA) and Pseudomonas Agar respectively (Hi-Media Laboratories, India). *E. coli* cultures were grown at 37°C while all *H. seropedicae* Z67 and *Pseudomonas* cultures were grown at 30°C. For growth in liquid medium, shaking was provided at the speed of 200rpm. The plasmid transformants of both *E. coli* and pseudomonads were maintained using respective antibiotics at the final concentrations as mentioned in (Table 2.2) as and when applicable. Both *E. coli* and *H. seropedicae* Z67 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.

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 2.2**).

Table 2.2: Recommended doses of antibiotics used in this study (Sambrook and Russell, 2001). The antibiotic doses 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.

2.2.1: Bushnell-Haas (BH) medium

The ingredients of BH agar include Ammonium nitrate, 1.0g/L; calcium chloride, 0.02g/L; dipotassium phosphate, 1.0g/L; ferric chloride, 0.05g/L; magnesium sulphate, 0.2g/L; monopotassium phosphate, 1.0g/L and agar, 20.0g/L. The readymade media was obtained from Hi-Media Laboratories, India, and was used according to manufacturer's instructions. Sterile sodium benzoate at the final concentration of 1mM was supplemented as the sole carbon source.

2.2.2: Koser's Citrate medium

Composition of the Koser's Citrate medium included magnesium sulphate, 0.2g/L; monopotassium phosphate, 1.0g/L; sodium ammonium phosphate, 1.5g/L; sodium citrate, 3.0g/L and agar, 15g/L. The readymade media was obtained from Hi-Media Laboratories, India, and was used according to manufacturer's instructions.

2.2.3: M9 minimal medium

Composition of M9 minimal broth was according to Sambrook and Russell (2001) including Na₂HPO₄ 7H₂O, 34g/L; KH₂PO₄, 15g/L; NH₄Cl, 5g/L; NaCl, 2.5g/L; 2mM MgSO₄; 0.1mM CaCl₂ and micronutrient cocktail. The micronutrient cocktail was constituted of FeSO₄.7H₂O, 3.5 mg/L; ZnSO₄.7H₂O, 0.16 mg/L; CuSO₄.5H₂O, 0.08 mg/L; H₃BO₃, 0.5 mg/L; CaCl₂.2H₂O, 0.03 mg/L and MnSO₄.4H₂O, 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.

2.2.4: HEPES buffered medium

The media composition included HEPES (pH=8.0), 100mM; NH₄Cl, 10mM; KCl, 10mM; MgSO₄, 2mM; CaCl₂, 0.1mM; micronutrient cocktail; Glucose, 100mM and phosphate (P) sources (Sharma et al., 2005). 1mg/ml Senegal Rock phosphate (RP) or KH₂PO₄ 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.

2.2.5: Pikovskaya's (PVK) Agar

The media composition included Ammonium sulphate, 0.5g/L; Calcium phosphate, 5.0g/L; Dextrose, 10.0g/L; Ferrous 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 readymade media was obtained from Hi-Media Laboratories, India, and was used according to manufacturer's instructions.

2.2.6: Murashige-Skoog's Medium

Murashige-Skoog's (MS) medium was composed of macro elements and micro elements. The macro elements included $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.440g/L; KH_2PO_4 , 0.17g/L; KNO_3 , 1.9g/L; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.37g/L; NH_4NO_3 , 1.65g/L and 10g/L agar. The micro elements included the essential trace elements. Micronutrients and desired carbon source (autoclaved separately), were added aseptically to reconstitute the complete media. The readymade micronutrient was obtained from Hi-Media Laboratories, India, and was used according to manufacturer's instructions while the macronutrients were reconstituted as and when required.

2.2.7: JNFb medium for *Herbaspirillum seropedicae* Z67

JNFb medium consists of l-malic acid, 5.0; K_2HPO_4 , 0.6; KH_2PO_4 , 1.8; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; NaCl , 0.1; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.02; trace element solution ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.2 g; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.235 g; H_3BO_3 , 0.28 g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.008 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.024 g; distilled water, 1000 ml), 2.0 ml; bromthymol blue (0.5% aqueous solution (dissolve in 0.2N KOH)), 2.0 ml; FeEDTA (1.64% solution), 4.0 ml; vitamin solution (biotin, 0.01 g; pyridoxine, 0.02 g; distilled water, 1000 ml), 1.0 ml; KOH, 4.5; pH adjusted to 5.8 with KOH. Bring the final volume to 1000 ml with distilled water. For a semisolid medium, add 1.75 to 1.9 g agar/l (agar should be dissolved before distribution into vials); for a solid medium, add 15.0 g agar/l. (Baldani et al., 1996)

2.3: Molecular biology tools and techniques

2.3.1: Isolation of plasmid and genomic DNA

2.3.1.1: Plasmid DNA isolation from *E. coli* and *Herbaspirillum seropedicae* Z67.

The plasmid DNA from *E. coli* and *H. seropedicae* Z67 was isolated by the boiling alkali lysis method, (Sambrook and Russell, 2001). Engineering the glucose metabolism of *H. seropedicae* Z67, by heterologous expression of and citrate synthase (CS) genes, PQQ gene clusters and GAD operon.

2.3.1.2: Isolation of large size plasmid DNA from *Herbaspirillum seropedicae* Z67

The large size of ~117kb TOL plasmids of *H. seropedicae* Z67 was isolated, by the protocol described for the isolation of plasmid (Ramos-Gonzalez et al., 1991). Single colonies of the *Herbaspirillum* were inoculated in 3ml of LB and were allowed to grow at 30°C under shaking conditions (200rpm). 0.5ml of freshly grown cultures was centrifuged at 9, 200xg for 2 minutes and the pellet was re-suspended homogeneously in 200µl of sterile STE[20% sucrose in 25mM Tris-Cl containing 25mM EDTA (pH=8.0) and 1mg/ml lysozyme]. Complete lysis of the cells was achieved by adding 100µl of alkaline SDS[0.3N NaOH containing 2% SDS]. After gentle mixing, the resulting viscous solution was incubated at 55°C for 5 minutes to minimize the chromosomal DNA contamination. Following this the solution was treated with equal volume of phenol-chloroform and after gentle mixing was subjected to centrifugation at 9, 200x g for 10 minutes. The aqueous layer recovered was subjected to chloroform extraction, centrifugation at 9,200x g for 10 minutes, recovery of the aqueous phase and finally DNA precipitation using 0.6 volumes of isopropanol. The DNA was allowed to precipitate at room temperature for ~30-40 minutes, washed with 70% ethanol and finally dissolved in 30µl Tris-EDTA (TE) buffer (Sambrook and Russell, 2001). Of this, 15µl DNA solution was subjected to agarose gel electrophoresis using 1% agarose (Section 2.4.4).

2.3.2: Transformation of plasmid DNA

2.3.2.1: Transformation of plasmid DNA in *E. coli*

The transformation of plasmids in *E. coli* using MgCl₂-CaCl₂ method and blue/white selection of the transformants using IPTG and X-Gal (as and when applicable) was carried out according to Sambrook and Russell (2001).

2.3.2.2: Electroporation of plasmid DNA in *Herbaspirillum seropedicae* Z67

Plasmid transformation in *Herbaspirillum seropedicae* Z67 was done by electroporation as described by (Unge et al., 1998). *H. seropedicae* Z67 cells grown till early stationary phase, cells were harvested (at 4°C) in sorvall washed 4 times with sterile distilled water and resuspended at 10¹⁰ cells ml⁻¹. Plasmid DNA of 0.5 µg µl⁻¹ was added in competent cells in electroporation cuvette. After 10 min of incubation on ice, the DNA was electroporated into cells using Eppendorf Electroporator system. System was adjusted to 2.5 kV and 186 Ω. The cells were immediately transformed to 1 ml LB and incubated for 45 min at 30°C, and then cells were plated on respective antibiotic for the selection of plasmid.

2.3.3: 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 1 µg/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.

2.3.4: Restriction enzyme digestion analysis

0.5-1.0 µg 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 10 µl. 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.

2.3.5: 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.

2.3.6: 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.

Amount of DNA (μg) $\times 1,515$

pmoles of DNA = -----

Size of the DNA fragment (no. of base pairs)

2.3.7: 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 2.6.

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)

Table 2.3: PCR conditions used in the present study.

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: Aromatic hydrocarbon utilization

Aromatic hydrocarbon utilizing ability of *Herbaspirillum seropedicae* Z67 strain was checked by monitoring the growth at 30°C on BH Agar (Section 2.2.1) containing 1mM of Na-benzoate as sole carbon source. Kanamycin was used at the final concentration of 7.5µg/ml. Accompanying phenotype of green fluorescence (attributed to expression of GFP) was monitored under UV light.

2.5: Lactose and Citrate utilization

Ability of the *Herbaspirillum seropedicae* Z67 to utilize citrate as carbon source and inability to assimilate lactose (Lessie and Phibbs, 1984) was exploited as a selection tool for pseudomonads in several experiments. Growth at 30°C on Koser's Citrate medium (Kiyohara et al., 1982) and M9 minimal medium containing 100mM lactose agar plates was monitored to indicate citrate and lactose utilizing abilities.

2.6: P-solubilization phenotype

Fresh culture of *H. seropedicae* Z67 transformants 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 characterize P-solubilizing ability of the native as well as the *H. seropedicae* transformants were monitored for (i) di-calcium phosphate (DCP) solubilization on Pikovaskya's (PVK) agar (Pikovskaya, 1948), (ii) HEPES buffered

RP-Methyl red as pH indicator dye and 1.5% agar (HRP) agar plates. HRP medium is similar to TRP medium with 50 mM HEPES pH 8.0 was used instead of 100 mM Tris pH 8.0 (Gyaneshwar et al., 1998). Culture was aseptically spotted on the HRP agar plates and was allowed to dry completely followed by incubation at 30°C for 2-4 days. P solubilization was determined by monitoring the zone of clearance on PVK agar and red zone on the HRP Methyl red agar plates.

2.7: Potassium solubilizing assay.

Fresh culture of *Hseropidecae* Z67 transformants 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 characterize K-solubilizing ability of the native as well as the *H. seropidecae* transformants were monitored for (i) Feldspar (Aleksandrov agar plate) (Hu et al., 2006), (ii) HEPES buffered Rock Phosphate and Feldspar - Methyl red as pH indicator dye and 1.5% agar (HRPF) agar plates. HRPF medium is similar to TRP medium with 50 mM HEPES pH 8.0 was used instead of 100 mM Tris pH 8.0 and Feldspar was used with Rock phosphate (Gyaneshwar et al., 1998). Culture was aseptically spotted on the HRPF agar plates and was allowed to dry completely followed by incubation at 30°C for 2-4 days. K solubilization was determined by monitoring the zone of clearance on Aleksandrov agar plate and red zone on the HRPF Methyl red agar plates.

(Aleksandrov agar medium constituted 1% glucose, 0.5% Yeast extract, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0005% FeCl_3 , 0.01% CaCO_3 , 0.2% CaPO_4 and 0.5% Feldspar, 3 % agar) .

2.8: Mutant Complementation Phenotype.

E. coli cs (W620) mutants were used to confirm the functionality of the cloned *cs* genes, respectively. The recombinant plasmids containing *cs* genes independently under *lac* promoter were transformed into respective mutants. The *cs* transformants were selected on streptomycin and kanamycin. These transformants were grown on M9 minimal medium with 0.2% (11.11mM) glucose as carbon source in presence and absence of 340µg/ml sodium glutamate, under shaking conditions at 30°C for *E. coli*

W620 at 37°C for. Kanamycin, and streptomycin were used at the final concentrations of 12.5µg/ml, 7.5µg/ml and 2.5µg/ml (1/4th of the concentration used for growth in LB broth). Both *ppc* and *cs* mutants exhibited glutamate auxotrophy (Izui et al., 1986; Underwood et al., 2002) as they could not grow on glucose as the sole carbon source in the absence of glutamate supplementation.

2.9: Physiological experiments

The physiological experiments were carried out using various WT and transformant pseudomonads which included growth, pH profile and enzyme assays.

2.9.1: Inoculum preparation

The inoculum for M9 and HEPES minimal media containing rock phosphate were prepared by growing the *H. seropedicae* Z6 overnight at 30°C in 3ml LB broth. Inoculum for the buffered RP broth (HRP) was prepared by growing the *H. seropedicae* Z6 overnight at 30°C in 10ml 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.

2.9.2: Growth characteristics and pH profile

Growth parameters and pH profile of the native as well as *H. seropedicae* transformants were determined using HRP medium with RP as P source (Gyaneshwar et al., 1998). Growth curve and pH profile was carried in 150ml conical flasks containing 30ml of relevant media. The initial O.D.600nm was about 0.09-0.15. The batch culture studies were performed under aerobic conditions in Orbitek rotary shaker maintained at 30°C with agitation speed kept constant at 200 rpm. 1ml samples were aseptically harvested at regular intervals and were subjected to analytical techniques. Microaerobic condition was maintained in flask by addition of ¾ volume of medium in 150 ml flask as per the method of Boesten and Priefer (2004). Flasks were sealed with rubber septum and incubated at 30°C by shaking at 200 r.p.m. and the syringe was used to take out samples.

2.10: Estimation of Plant growth promoting factors

2.10.1: Culture conditions for EPS production and quantification

Fresh overnight grown culture of *Hs* Z67 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 4°C for 2h, EPS obtained in each extraction were separated by centrifugation from solution and under 4°C. 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.

2.10.2: Biofilm 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).

2.10.3: Indole acetic acid (IAA) production and estimation

Overnight grown all cultures were pulled in sterile 1.5ml eppendorf in laminar, asptic 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 30°C 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 $9000 \times g$ 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 FeCl_3 + 50 ml of 35% perchloric acid) was added and incubated for 30 mintues under darkness. Pink colour formed was determined colorimetrically at 540 nm as IAA production.

2.11: PQQ determination

PQQ production was estimated using the method of Rajpurohit et al., (2008). *Hs Z67* transformants and other bacteria were grown for 36 h on M9 minimal medium containing 50 mM glucose. Cell supernatant was digested with 50% acetonitrile at 65°C for 2 h. The mixture was centrifuged at 15,000 g for 10 min; the clear supernatant was collected and dried with a concentrator under a vacuum. The residue was dissolved in 50% *n*-butanol at 1 mg/ml, and PQQ was extracted at 50°C overnight. The clear supernatant was dried under a vacuum and dissolved in 100% methanol The identity of the PQQ was ascertained by comparing the with standard PQQ on spectrofluorometer Fluorescence was monitored at *ex* 360 and *em* 480 nm.

2.12: Analytical techniques

Modified Lowry's method (Peterson 1979) was used for total protein estimation. *H. seropedicae* Z67 native as well as transformants increased in cell growth density was determined at OD600 (spectrophotometer). Increase in culture

growth, pH drop indicates organic acid secretion and phosphate solubilisation from rock phosphate in medium, End point culture supernatants samples were filtered through 0.2 µm nylon membranes further they were used for Phosphate estimation by the ascorbate method (Ames 1964) same samples were used for HPLC analysis to detect organic acid levels, retention time of acids were determined under above mentioned conditions. Standards organic acids were used for quantification (equipment from Shimadzu , India) HPLC was performed using a C18 column operated at room temperature with 0.01 M H₂SO₄ as mobile phase at a flow rate of 0.7 ml min⁻¹, while for citric acid estimation the same column was operated at room temperature using mobile phase of 20mM Na₂HPO₄ with 2.5% acetonitrile at a flow rate of 1.0 ml min⁻¹ and the column effluents were monitored using a UV detector at 210 nm (Buch et al., 2009, 2008) and measuring left over glucose using the GOD-POD kit (Enzopak, Reckon Diagnostics Pvt. Ltd., India). Physiological parameters were calculated like growth rate, biomass yield on glucose and specific glucose reduction rate as described by (Buch et al., 2009, 2008). Graph Pad Prism (version 3.0) and Microsoft Excel were used for statistical analysis of the parameters. Each parameter has been represented as mean ± SD or mean ± SEM as specified in the figure legends.

(i) Specific growth rate (h⁻¹):

$$k = \frac{(\text{Log}_{10}N_{t1} - \text{Log}_{10}N_{t2}) \times 3.3}{(t_1 - t_2) \text{ (h)}} \quad \text{where,}$$

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):

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

$\Delta\text{Glucose (t1-t2)}$ is the amount of glucose consumed over the time interval t1-t2; $\Delta\text{dcw (t1-t2)}$ 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

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

All the parameters were as described for Specific glucose utilization rate. $Y_{\text{dcw/Glc}}$ is expressed as g of dry cell weight produced/ g glucose utilized/h.

(iv) Organic acid yield

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

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)

2.13: Enzyme assays

2.13.1: 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, citrate synthase (CS), the preparation of cell free extracts for, CS, assays was carried out according to Kodaki et al (1985) The cell pellet was washed once with 80 mM phosphate buffer (pH=7.5) followed by resuspension in same buffer containing 20% glycerol and 1mM 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 phosphate buffer (pH 6.0) with 5mM MgCl₂.

2.13.2: Enzyme Assay Protocols

2.13.2.1: 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 600 nm (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 3.0 ml.

2.13.2.2: Gluconate dehydrogenase assay (GADH)

Hs natives as well as transformants were grown on M9 and RP-containing minimal medium with 50 mM D-gluconate as the C source, Additionally RP-containing

minimal medium contains 50mM HEPES buffer, pH 8.0, Cells were harvested (5000g for 10 min) after the pH dropped below 5.5, washed with sterile saline, and resuspended in 50 mM Tris-HCl, pH 8.75, and the whole-cell suspension was used as the source of enzyme in GADH assays. Spectrophotometrically method was followed for estimation of GADH activity according to Matsushita et al., (1982). Substrate of the GADH enzyme was done with D-gluconate, in the assay mixture. GADH activity was defined as nanomoles of 2, 6-dichlorophenolindophenol (DCIP) reduced per minute using D-gluconate, as substrates, respectively. Enzyme activity is defined as one unit of specific enzyme activity was defined as the amount of protein required to convert one nanomole of substrate per minute per milligram of total protein.

2.13.2.3: 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 $13.6 \text{ mM}^{-1}\text{cm}^{-1}$ at 412 nm. 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,

ΔA_y nm is the difference in the absorbance at any given wavelengths (y nm) and ϵ 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.

2.14: Hydroponic plant study with rice. (*Oryza sativa*).

The hydroponic experiment was designed in three replications according (Crestani et al., 2009). Rice (*Oryza sativa*, Gujarat-17) seeds were surface sterilized in 70% ethanol for 1 min and in 1% sodium hypochlorite (NaClO) for 20 min, and then rinsed 5- 6 times with sterile distilled water and then allowed to germinate for 72 h on moist filter paper in petriplates kept in the dark at 30°C placed Uniform grown seedlings with 5 mm radicles were transferred to nylon nets placed on top of beakers containing 500 ml hydroponics nutrient solution for rice suggested by (Miyamoto et al., 2001), Stress of cadmium (CdCl_2) in μM and sodium chloride (NaCl) in mM was added independently in hydroponic containing nutrient medium. Exogenously PQQ of 100 nM was also added independently to set of stress given plants (Choi et al., 2008). After 10 day of treatment plants were removed and rinsed with tap water and used for catalase assay, superoxide dismutase assay, root length and shoot length was measured to know the effect of stress on rice plants.

2.15: Pot experiments - Interaction with (*Oryza sativa* Gujarat-17).

2.15.1: Plant Inoculation Experiments:

Pure bacterial cultures were grown in nutrient broth at 30°C, centrifuged, and diluted to a final concentration of 10^8 CFU/ml in sterile distilled water. Rice (*Oryza sativa*, Gujarat-17) seeds were surface sterilized in 70% ethanol for 1 min and in 1% sodium hypochlorite (NaClO) for 20 min, and then rinsed 5- 6 times with sterile distilled water and then allowed to germinate for 24 h on moist filter paper in petriplates kept in the dark at 30°C and then immersed in a bacterial suspension for 5 h. Germinated seeds were sown in plastic bags filled with 1Kg of autoclaved, locally collected soil. Before sowing seeds in bags initial physical and chemical properties in experimental of soil is presented in (Table.4). The plants were grown at 30°C under natural day light and were irrigated with 50 mL of sterile distilled water every day.

The experiment was conducted on six replicates (three bags per replicate, two plants per bag) for each treatment and was completely randomized. The plants were harvested after 30 days and growth parameters (plant height and plant biomass) were recorded. Total N_t was measured by micro Kjeldahl's method, by Vepodest-Automatic Digestion, Distillation and titration system. Total P content was measured by Vanadomolybdo phosphoric acid yellow color method, and Total K content was measured by Flame photometer.

Table 2.4: Physical and chemical properties of experimental soil used for *H. seropedicae* Z67 transformants

E.C	pH	Total phosphorus (%)	Total nitrogen (%)	Total potassium (%)
0.63	7.86	19	16	65

2.15.1.1: Antioxidant Enzymes / ROS scavenging enzyme activity:

(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) polyvinylpyrrolidone (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 tetrazolium (NBT). The 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 μ M NBT, 2 μ M 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).

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

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

(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) polyvinylpyrrolidone (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 ($\epsilon = 39.4 \text{ mM}^{-1} \text{ cm}^{-1}$) got consumed according to the method of Aebi (1984) and enzyme activity expressed as $\text{mmol H}_2\text{O}_2 \text{ oxidized min}^{-1} \text{ g}^{-1} \text{ protein}$. 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:

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

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

2.15.1.2: Estimation of Water Soluble Protein Content

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

(a) Reagents for Lowery'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 & Ciocalteu reagent (1N).

(b) Procedure (Folin Lowery'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 & Ciocalteu 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.