Chapter 3
Materials and Methods

3.1. The study area

The northern province of West Bengal i.e. North Bengal and Sikkim were the area of germplasm collection.

3.2. Collection of germplasm

Different places of North Bengal and Sikkim were visited for the collection of germplasm. The places included Darjeeling, Kurseong, Kalimpong, Sonada, Mirik, Bijanbari, Siliguri, CoochBehar, Raiganj, Malda, Balurghat, Samsing, Daramdin, Gangtok and Mangan (for details about collection sites refer Table 3.1and Fig. 3.1).

The nodulated roots of healthy French bean (*Phaseolus vulgaris*) plants (host plants) were collected from different regions in their respective growing season. Essential data were recorded during the collection of germplasm (for details see Fig. 3.2). In some cases the nodules were collected directly and preserved in desiccated silica gels. The vials containing the collected nodules were refrigerated at 4 ºC until further use.

3.3. Isolation of *Rhizobium*

3.3.1. Surface sterilization

Prior to isolation experiment, the surface sterilization of root nodules was performed.

At first, the nodulated roots were washed thoroughly with tap water to remove all the soil and organic materials. Healthy and undamaged nodules were selected from the root and washed with few drops of detergent (Dextran) followed by washing with distilled water. The nodules were then treated with 70% ethanol for 1 min followed by several washes with sterile distilled water. The nodules were further treated with 30% hydrogen peroxide (H_2O_2) for 2 min. Final rinsing of the nodules was done thoroughly with sterile distilled water for several times to remove the traces of H_2O_2.

Table 3.1. Geographical locations from where germplasm were collected

Sl. #	District	Site name	Strain Code	Altitude (ft)	Latitude	Longitude
1	Darjeeling		MTCC 99*			
2		Kurseong	K-1	4,873	26.8778° N	88.2772° E
3		Darjeeling	DJG	6,700	27.0500° N	88.2667° E
4		Sonada	RSm-3	6,143	26.9400° N	88.250° E
5		Kalimpong	KPG-5N	4,101	27.0600° N	88.4700° E
6		Mirik	MIR-6	4,902	26.8855° N	88.1873° E
7		Bijanbari	BIJ	2,490	27.0400° N	88.1100° E
8		Siliguri	NBU-8	399	26.7100° N	88.4300° E
9		Siliguri	RSv-1	399	26.7100° N	88.4300° E
10	Cooch Behar	Cooch Behar	CBR	121	26.3242° N	89.4510° E
11	Jalpaiguri	Samsing	SAM-12	3000	26.5600° N	88.4800° E
12		Jalpaiguri	JPG	246	26.7000° N	89.0000° E
13	Malda	Malda	RMa-13	56	25.0000° N	88.1500° E
14	South Dinajpur	Balurghat	BLG	82	25.2200° N	88.7600° E
15	North Dinajpur	Raiganj	RGJ	130	25.6200° N	88.1200° E
16	North Sikkim	Mangan	SKM-N	3,136	27.5200° N	88.5300° E
17	West Sikkim	Daramdin	SKM-W	4,088	27.2170° N	88.1710° E
18	East Sikkim	Gangtok	SKM-E	5,410	27.3300° N	88.6200° E

*Reference strain

3.3.2. Media for isolation

Yeast extract mannitol (YEM) medium was used for the isolation of *Rhizobium.*

3.3.3. Isolation of Rhizobium in liquid and solid media

After proper sterilization, the isolation procedure were carried as follows:

- Surface sterilized nodule lobes were crushed on a grease free sterile slide.
- A loopful of the crushed nodule sap was then put into liquid YEM medium.
- The incubation was done in an orbital shaker at 200 rpm maintained at an optimum temperature of 28 ± 2ºC for 2-3 days. The media turned to be turbid with the growth of the bacteria.
- A loopful of culture was streaked on YEM agar plates containing 0.0025% (w/v) Congo red (Vincent, 1970).
- The YEMA plates were incubated at 28±2ºC for 2-3 days.
- The single pure colony obtained was then sub cultured and maintained in YEM slants for further use. The strains were sub cultured at regular interval of one month.

The pure strains isolated from different regions were then named with their

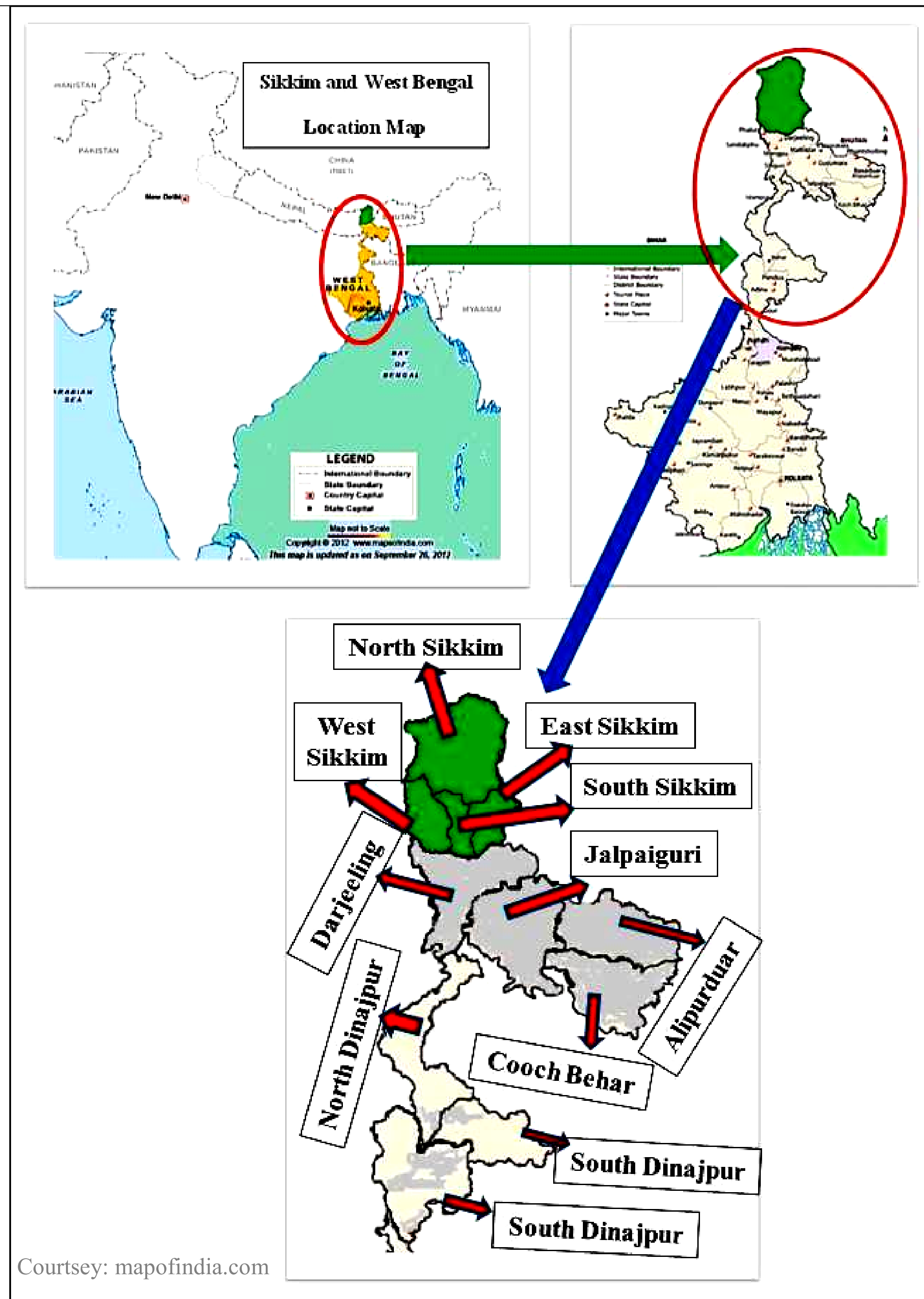

Figure 3.1: Maps showing collection sites

respective codes (for details about strain codes please refer Table 3.1). The reference strain *Rhizobium leguminosarum* MTCC 99, procured from Institute of Microbial Technology, Chandigarh was used as a positive control.

3.4. Plant Infectivity Test

The pure culture of Rhizobial strains were subjected to Koch's postulation to check the authenticity of isolates.

COLLECTION DATA SHEET

SAMPLE NO.: K-1

A. GENERAL INFORMATION

1. Collection site and State: Kurseong, West Bengal
2. Date of collection: 14.01.14
3. Scientific name: *Phaseolus vulgaris*
4. Local name: Sini
5. Nodules Present/Absent
6. Nodules collected: Yes/No
7. Root collected: Yes/No
8. Seed collected: Yes/No
9. Soil collected: Yes/No

B. HABIT

1. Herb/Shrub/Tree: Climber
2. Flowering time: July - August
3. Fruit setting time: October
4. Planting time: April - May

C. HABITAT AND AREA OF THE VEGETATION

1. Rainfall season: June - September
2. Altitude: 4,873 ft
3. Topography: Swamp/Plain/Hilly/Mountain/Other
4. Vegetation Type: Natural Forest/Cultivated Land
5. Management: Cutting/Burning/Natural/Agriculture

D. SOIL TYPE

1. Soil Texture: Sandy/Loamy/Clayey/Organic/Rocky/Sandy Loamy
2. pH:
3. Colour: Red/Yellow/Brown/Grey
4. Drainage: Flooded/Poorly Drained/Well Drained

E. NODULES

1. Location: Crown Area/Tap Root/Lateral Root
2. Growth Form: Clumped/Scattered/

COLLECTED BY: Ritu Rai

SIGNATURE 14/01/14

Figure 3.2: Sample Collection Data Sheet

3.4.1. Surface sterilization of seed

- Firstly, healthy French bean seeds were soaked in water for nearly 1 hr to remove the toxic substances adhered to the seed.
- Thereafter, the seeds were surface sterilized with 0.1% (w/v) $HgCl_2$ for 2 minutes, followed by several washing with sterile distilled water. The seeds were then dried using a tissue paper.

3.4.2. Seed germination

- The seeds were placed on sterile water agar medium plates and kept in the tissue culture room in an aseptic condition for 16 hr photoperiod under artificial light for germination.
- After 8-10 days, the germinated saplings were transferred to an improvised Leonard jar for the Koch's postulation.
- About 2 litre of an empty plastic bottle was cut in the middle and the upper part was placed upside down in the lower half.
- The lower part of the bottle was filled was sterile modified nitrogen free Jensen's liquid medium (please refer appendix C for composition) whereas the upper half was filled with approximate 250-300 gm of sterile sand.
- Ten days old seedlings were then planted on the sand in an aseptic condition.
- At the end of the log phase, bacterial cultures were centrifuged (REMI make, Model No.C-24) at 5,000 Xg for 15 min at 25ºC and the supernatant was decanted.
- The pellet was scraped into sterile distilled water and an aqueous suspension was made.

The following three sets were prepared for each isolate:

- Seedlings inoculated with 100µl of 2 days old reference strain (+ve control).
- Seedlings inoculated with 100µl of 2 days old *Rhizobium* pure culture under test.
- Uninoculated seedlings (-ve control).

The seedlings were allowed to grow in the culture room. After 30-45 days, the Leonard jars were dismantled and the roots were visually observed for any nodule formation.

The size of the nodules were measured. The bacteria was again isolated from the nodules obtained via. Koch's postulation and grown in YEM slants as discussed in section 3.3.2. for further characterization .

3.5. Microscopy

3.5.1. Light microscopy

One loopful of fresh culture was taken on a grease free slide. The staining was performed as per the standard Gram staining procedure (Aneja, 1996) and finally the strains were observed

under light microscope using oil immersion.

3.5.2. Scanning Electron microscopy

- For scanning electron microscopy of the bacterial cells, 2 days old culture grown in nutrient broth medium were centrifuged at 5000 Xg.
- The pellet was collected and washed with 0.1M phosphate buffer saline.
- Samples were tmaneuveringhen prefixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) under vacuum, followed by dehydrolysis of the sample treating with a series of ethanol (30%, 50%, 70%, 80%, 90% and 100%) for 10 minutes each.
- After stepwise dehydration in graded alcohol, the samples were 'critical point dried' in CO_2 (CPD 030; BAL TEC, Vaduz, Liechtenstein) mounted onto the sample stubs and were coated with 20 nm silver-palladium alloy in a mini sputter coater (SC7620) and examined in a Carl Zeis Scanning Electron Microscope.

The Scanning Electron microscopy was done at The Energy and Resources Institute (TERI), Gurgaon, Haryana.

3.6. Morphological characterization

Fresh 24-48 hr cultures were used for morphological and biochemical analysis.

3.6.1. Colony morphology

A loopful of fresh culture from broth was streaked on the YEMA plates and incubated for 24-48 hr at 28 ± 2ºC. The morphology of the colonies were examined based on the diameter, shape, colour, transparency, form and production of mucous (Aneja, 1996).

3.6.2. Gram staining

Gram-staining reaction was carried out as per the standard Gram's procedure (Aneja, 1996).

The isolates were observed under oil immersion using a light microscope to differentiate gram negative from the gram positive bacteria.

3.7. Biochemical characterization

3.7.1. Hofer's alkaline medium

The fresh cultures were grown in Hofer's alkaline medium (refer appendix C for composition) using plates with the pH adjusted at 11 (Hofer, 1935). The growth was checked after 24-48 hr.

3.7.2. Ketolactase test

In this test, the isolates were streaked

on lactose agar medium (refer appendix C for composition).

The plates were incubated for 2-3 days at 28±2ºC. Five milliliters of Benedict's reagent was poured on the plates and kept at room temperature for 1 hr (Bernaerts and De Ley, 1963).

3.7.3. Acid & alkali production test

The production of acid and alkali was detected by allowing the isolates to grow on YEM medium with bromothymol blue (BTB) (1.5 ml/100ml).

The freshly grown isolates were inoculated on stab containing YEMA with BTB and incubated at 28 ± 2ºC for 24-48 hr. The color change of the media was recorded (Somasegaran and Hoben, 1994).

Similarly, the inoculation was also done on YEM with BTB broth and incubated in the shaker at 200 rpm at 30ºC for 24-48 hr. The pH of the media was then measured with the pH meter (Lab India).

3.7.4. Glucose peptone agar (GPA) test

Fresh 24 hr culture was streaked on GPA medium (refer appendix C for composition) plates followed by incubation at 30ºC for 24-48 hr and the growth was observed (Singh *et al.* 2008).

3.7.5. Methylene blue test

In this test 0.1% (v/v) methylene blue was added to YEMA medium and the rhizobial isolates were streaked on the medium and incubated for 24-48 hr (Singh *et al.* 2008). The presence or absence of the growth of the isolates was checked.

3.7.6. Starch hydrolysis test

The isolates were inoculated on Starch agar media (SAM) plates and incubated at 28 ± 2ºC for 24-48 hr. After incubation, drops of iodine solution were poured on surface of the plates and kept for 5-10 mins. The presence or absence of clear halos around the colonies was recorded (Aneja, 1996) .

3.7.7. Gelatin hydrolysis

The fresh grown cultures were inoculated in the nutrient gelatin medium (refer appendix for composition) stabs and was incubated at 30ºC for 24-48 hr. After incubation, the tubes were placed at 4ºC for 15 min. The liquefaction of the media was recorded (Aneja, 1996)..

3.7.8. Triple sugar iron (TSI) agar test

Freshly grown 24 hr cultures were

inoculated on TSI slants and incubated at 28 ± 2ºC for 24-48 hr. The change in the color of slant and butt was observed (Singh *et al*. 2008).

3.7.9. Production of Catalase

About 1 ml of 3% H_2O_2 was directly added on the fresh pure bacterial culture grown on YEM slant. The tube was placed against a dark background and observed for immediate effervescence (Cappuccino and Sherman, 2008).

3.7.10. Urease test

Urease test was performed by inoculating the fresh grown cultures on urea agar medium (refer C appendix for composition) with pH indicator phenol red (0.2% solution, pH 6.8) and incubated at 28 ± 2ºC for 2-7 days. (Aneja, 1996).

3.7.11. Cellulase production test

Fresh 24 hr cultures were streaked on Czapek-mineral salt agar medium (refer appendix C for composition) plates and incubated at 28 ± 2ºC for 24 -48 hr. After the incubation the plates were then flooded with 1% aqueous solution of HDTMA (Hexadecyl trimethyl ammonium bromide), a reagent that detects the microbial utilization of cellulose. The formation of clear zone around the colonies, if any was recorded (Aneja, 1996).

3.7.12. Nitrate Reduction test

The nitrate reduction test was determined by the method of (Cappuccino and Sherman, 2008)

A loopful of fresh culture was inoculated in the test tube containing YEM broth with 1% KNO_3 and incubated for 7 days at 28 ± 2ºC.

The broth cultures were then treated with one dropper full of sulphanilic acid (approx. 2 ml) and another dropper full of α-naphthylamine reagents and incubated for 48 hr. Thereafter, the color change of the solution was observed.

3.7.13. Carbohydrate utilization test

3.7.13.1.Using Phenol Red carbohydrate fermentation broth-

Fresh culture was inoculated in tubes containing nutrient broth with 0.1% sugar like glucose, fructose, dextrose, mannitol, sucrose, lactose. The tubes were incubated for 24-48 hr at 28 ± 2ºC. Few drops of phenol red was added and the color change was recorded to detect sugar fermentation by the bacteria.

3.7.13.2. Using MOPS-salt (MS) medium

The minimal media used for the carbohydrate utilization test was MOPS-salt (MS) medium (refer appendix C for composition) (Jordan, 1984).

- Stock solution of MOPS-KOH buffer was prepared and sterilized by filtration.
- The stock solution of salt and Magnesium sulphate ($MgSO_4$) was separately prepared and sterilized by autoclaving.
- Finally, MOPS-KOH buffer and $MgSO_4$ were added aseptically to the salt solution.
- All the carbon sources to be tested were sterilized by filtration and added to the medium making the final concentration of 15mM. However, in the case of citrate, 5 mM concentration was used (Jordan, 1984).
- Carbon sources like glucose, fructose, dextrose, mannitol, sucrose, lactose and some organic salts like sodium acetate, sodium succinate, sodium propionate, sodium citrate and sodium pyruvate were utilized for the test.
- The growth of isolates in different carbohydrate sources were scored in binary form and cluster analysis was done using NTSYSpc2 (Numerical Taxonomy analysis program package, Exeter software, USA) software package, version 2 (Rohlf, 1998). .
- The isolates were grouped by the unweighted paired group method using arithmetic means (UPGMA) and depicted in a dendrogram.

3.8. Physiological characterization

The *Rhizobium* strains were subjected to various NaCl, pH, temperature, heavy metal and antibiotics stresses to check their tolerance level.

3.8.1. Sodium chloride tolerance test

A loopful of fresh culture of *Rhizobium* strains were inoculated in 200 ml conical flask containing the growth medium YEM with 1, 2 and 4% (w/v) concentrations of sodium chloride (NaCl). The broth was then incubated at 28 ± 2ºC in OSI at 180rpm. After 48hr, the O.D values of the broth was recorded at 540nm in a spectrophotometer.

3.8.2. pH tolerance test

A loopful of fresh culture of *Rhizobium* strains were inoculated in 200 ml

conical flask containing the growth medium YEM adjusted to pH 4, 7 and 9. The broth was then incubated at 28 ± 2ºC in OSI at 180rpm. After 48hr, the O.D values of the broth was recorded at 540nm in a spectrophotometer.

3.8.3. Temperature tolerance test

A loopful of fresh culture of *Rhizobium* strains were inoculated in 200 ml conical flask containing the growth medium YEM and incubated at different temperatures like 10ºC, 20ºC, 30ºC and 40ºC. The broth was then incubated at 28 ± 2ºC in OSI at 180rpm. After 48hr, the O.D values of the broth was recorded at 540nm in a spectrophotometer.

3.8.4. Heavy metal sensitivity test

Rhizobium cultures were subjected to heavy metal stress to determine its sensitivity. The heavy metals used in the experiment were Cobalt chloride ($CoCl_2$), Lead nitrate ($Pb(NO_3)_2$) and Copper sulphate ($CuSO_4$).

- Different concentration of heavy metals were made from the stock solution(1M) like 0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM, 1 mM, 2 mM and 4 mM.
- A loopful of fresh culture was inoculated on YEM broth amended with different concentration of the heavy metals.
- The inoculated flasks were then placed at 28 ± 2ºC on a shaker at speed 180 rpm for 48 hr. The optical density of the cultures were measured at 540nm in a spectrophotometer.

The statistical analysis of the results of the OD values obtained from the NaCl, pH, temperature, heavy metal was done by the student's t-test and the plotting was done by KyPlot.

3.8.5. Intrinsic antibiotic resistance (IAR) test

3.8.5.1.Antibiotics

The experiment was carried out using two sets of multiple antibiotic discs: Icosa G-I-Minus (IC003) and Icosa-Universal-2 (IC006). Each antibiotic strips contained 20 different disc of antibiotics. The symbols and concentrations of the respective antibiotics are given in Table 3.2.

3.8.5.2. Procedure

The resistance of *Rhizobium* to different concentration of antibiotics was determined using agar disc diffusion system of Bauer *et al*. (1966).

- Freshly prepared YEMA was poured on 20 cm petriplate (approx.

Table 3.2. Disks used for the Intrinsic Antibiotic Resistance (IAR) test

Group	Antibiotics	Sybl.	Conc. (mcg)	Disk type
Aminoglycosides	Amikacin	Ak	30	Icosa-Universal-2(IC006)
	Amikacin	Ak	30	Icosa G-I-Minus (IC003)
	Gentamicin	G	10	Icosa-Universal-2(IC006)
	Gentamicin	G	10	Icosa G-I-Minus (IC003)
	Netilmycin	Nt	10	Icosa-Universal-2(IC006)
	Tobramycin	Tb	10	Icosa-Universal-2(IC006)
	Kanamycin	K	30	Icosa G-I-Minus (IC003)
	Streptomycin	S	10	Icosa G-I-Minus (IC003)
Amphenicol	Chloramphenicol	C	30	Icosa-Universal-2(IC006)
Carbapenem	Imipenem	I	10	Icosa G-I-Minus (IC003)
Cephalosporin	Ceftazidime	Ca	30	Icosa-Universal-2(IC006)
	Ceftriaxone	Ci	30	Icosa-Universal-2(IC006)
	Ceftriaxone	Ci	30	Icosa G-I-Minus (IC003)
	Cefadroxil	Cq	30	Icosa-Universal-2(IC006)
	Cefoperazone	Cs	75	Icosa-Universal-2(IC006)
	Cefpodoxime	Cep	10	Icosa G-I-Minus (IC003)
Glycopeptide	Vancomycin	Va	30	Icosa-Universal-2(IC006)
Hydantoin	Nitrofurantoin	Nf	300	Icosa-Universal-2(IC006)
Macrolide	Erythromycin	E	15	Icosa-Universal-2(IC006)
Penicillin	Ampicillin	A	10	Icosa-Universal-2(IC006)
	Amoxycillin	Am	10	Icosa-Universal-2(IC006)
	Cloxacillin	Cx	1	Icosa-Universal-2(IC006)
	Penicillin	P	10	Icosa-Universal-2(IC006)
	Augmentin	Au	30	Icosa G-I-Minus (IC003)
	Ticarcillin	Ti	75	Icosa G-I-Minus (IC003)
Polypeptide	Colistin	Cl	10	Icosa G-I-Minus (IC003)
Quinolones	Nalidixic acid	Na	10	Icosa-Universal-2(IC006)
	Norfloxacin	Nx	10	Icosa-Universal-2(IC006)
	Ciprofloxacin	Cf	5	Icosa-Universal-2(IC006)
	Ciprofloxacin	Cf	5	Icosa G-I-Minus (IC003)
	Moxifloxacin	Mo	5	Icosa G-I-Minus (IC003)
	Plixacin	Of	5	Icosa G-I-Minus (IC003)
	Sparfloxacin	Sc	5	Icosa G-I-Minus (IC003)
	Levofloxacin	Le	5	Icosa G-I-Minus (IC003)
	Norfloxacin	Nx	10	Icosa G-I-Minus (IC003)
	Nalidixic acid	Na	30	Icosa G-I-Minus (IC003)
	Nalidixic acid	Na	30	Icosa G-I-Minus (IC003)
	Gatifloxacin	Gf	5	Icosa G-I-Minus (IC003)
Sulfonamide	Co-Trimoxazole	Co	25	Icosa G-I-Minus (IC003)
	Co-Trimoxazole	Co	25	Icosa-Universal-2(IC006)

150 ml) each and was allowed to cool down for solidification.

- Approximately 2 ml of fresh culture of *Rhizobium* strains were poured at the centre of the agar medium.
- The culture was spread evenly on the agar medium with a sterilized cotton swab to make a bacterial lawn.

- Each impregnated antibiotic discs were placed onto the agar surface adjusting the distance from the center.
- The plate was then left drying for 4 -5 min. The plates were incubated at 28 ± 2ºC for 24-48 hr in an inverted position.
- The diameter of each zone of inhibition was recorded and then compared with a standard value of CLSI (formerly NCCLS).

3.8.5.3. Statistical analysis

The statistical analysis of the antibiotic resistance was done with the gplot package of R-statistical software generating a heatmap (http://www.r-project.org). The color package topo. colors (256) was used for the color pattern.

All the chemicals used in these experiments were purchased from Hi-media, Mumbai, India unless otherwise mentioned. All the experiments were conducted in triplicates.

3.9. Screening of plant growth promoting activity

The eighteen *Rhizobium* strains were subjected to different tests to study their PGPR (Plant Growth promoting rhizobacteria) activity

3.9.1. Study of *in vitro* PGPR activity

3.9.1.1 Phosphate solubilization test

The test was performed following the protocol of Pikovskaya (1948).

A loopful of freshly grown pure culture was spot inoculated on Pikovskaya's agar medium. The plates were incubated at 28 ± 2ºC for 3-5 days.

The presence or absence of clear transparent zone around the colonies were recorded. The solubilization zone was calculated by substracting the diameter of bacterial colony from the diameter of total zone.

The Phosphate solubilizing efficiency (PSE) was calculated with the following formula:

$$PSE(\%) = \frac{Z - C}{C} X100$$

where, Z= diameter of halo zone, C=diameter of the colony

3.9.1.2. Siderophore production

The siderophore production test was performed following the method of (Alexander and Zuberer, 1991) using Chrome- azurol S(CAS) agar medium.

For the purpose following 4 solutions were prepared:

(i) Fe-CAS indicator solution (Solution 1)-

- Solution 1 was prepared by mixing 1 mM Ferric chloride ($FeCl_3.6H_2O$) in 10 ml of 10 mM HCl with 50 ml of an aqueous solution of CAS (1.21 g/l) resulting to a dark purple colored mixture.
- The mixture was then added slowly to 40 ml of an aqueous solution of HDTMA (Hexadecyl trimethyl ammonium bromide, HiMedia) (1.82 g/l) with a constant stirring.
- The final dark blue mixture was autoclaved and cooled to 50°C.

(ii) Buffer solution (Solution 2)-

- The solution was prepared by dissolving 30.24 gm PIPES (1,4-Piperazinediethanesulfonic acid) in 750 ml of a salt solution containing 0.3 g K_2HPO_4, 0.5 g NaCl and 1 g NH_4Cl.The pH of the solution was adjusted to 6.8 with 50% KOH.
- Finally water was added to bring the final volume to 800 ml.
- 15 g of agar was added and then autoclaved and cooled to 50°C.

(iii) Nutrient medium (Solution 3)-

YEM medium was used as the nutrient medium for growth of the isolates.

(iv) Casamino acid (Solution 4)–

Thirty ml of Millipore filter sterilized 10% casamino acid was prepared as solution 4.

After the preparation of the four solutions, Solution 2 was mixed with solution 3 and 4 and the mixture was added in solution 1 (Fe-CAS indicator solution).

- The finally prepared blue colored CAS agar medium was then poured on the sterilized petriplates.
- A loopful of pure *Rhizobium* culture grown for 24-48 hr was spot inoculated on CAS agar medium plates and incubated for 2 -5 days at 28 ± 2ºC.

3.9.1.3. Indole Acetic Acid production

The IAA production test was followed as per Bric *et al*. 1991.

- Yeast extract mannitol medium was supplemented with filter sterilized solution of 0.1% L-tryptophan. The broth was inoculated with loopful of fresh *Rhizobium* culture and incubated at 200 rpm at 28 ± 2ºC for 24-48 hr.
- The fully grown bacterial cultures were then centrifuged at 7000Xg for 10 min at 4ºC.
- Two ml of supernatant was then mixed with 4 ml of Salkowski

reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml 35% perchloric acid).

- The absorbance of the resultant pink colour was read at 535 nm in UV/visible spectrophotometer.
- The IAA production was calculated from the regression equation of standard curve and the result was expressed as µg/ml over control (Gordon and Weber, 1951).

3.9.1.4 Hydrogen cyanide production

The hydrogen cyanide production was determined as per the protocol of Castric(1975).

- A loopful of fresh bacterial was streaked on YEMA medium amended with glycine(4.4g/l).
- A sterilized Whatman filter paper No.1 was soaked in 0.5% picric acid solution amended with 10 % sodium carbonate and dried.
- The dried filter paper was placed inside the lid of a plate which was sealed with parafilm. The plates were then incubated at 28±2ºC for 3-4 days.

3.9.1.5. Ammonia production test

Ammonia production was detected by the method of Cappucino and Sherman (1992).

- The prepared peptone water were distributed in test tubes and sterilized.
- Fresh 1 ml culture was inoculated and incubated at 28±2ºC for 24-48 hr.
- About 0.5 ml of Nessler's reagent was added and the color change was recorded.

3.9.1.6. Antagonistic activity against fungal pathogen

The *Rhizobium* strains under study were tested *in vitro* for their antagonistic activity against the fungal root pathogen *Fusarium solani*. The fungus *F. solani*(RHS/P388) with accession number NAIMCC-F-02901 was obtained from the culture collection maintained at Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal.

- The *in vitro* antagonism test was performed on potato dextrose agar (PDA) by dual culture method. (Arfaoui *et al*. 2006).
- Firstly, the pure culture of fungus was grown on PDA medium for 7 days.
- From the fully grown fungal pathogen culture plate, a 5 mm

mycelial disc of the fungal pathogen was bored by a cork borer and then placed on one side of a petridish containing fresh PDA medium.

- A loopful of fresh bacterial culture was streaked 5 mm away from the fungal pathogen.
- The plate was then sealed tightly with a parafilm and incubated at 25ºC -30ºC for 5-7 days.
- Petridish containing PDA medium inoculated only with the fungal pathogen *Fusarium solani* served as a control.

The test was performed in triplicate to avoid any error in the results.

3.9.2. *in vivo* study of the selected strains showing PGPR traits

The potential *Rhizobium* strains showing the PGPR activity were selected to be used as biofertilizers

3.9.2.1. Biofertilizer preparation

- Preparation of the inoculum culture- A loopful of two to three days old culture of the potent *Rhizobium* strains showing PGPR activity was grown in YEM with shaking at 200 rpm for 48 hr at 28±2°C.
- Carrier for rhizobial inoculants- In this present study, charcoal was used as a carrier for *Rhizobium* inoculants due to its easy availability and the equivalent performance to the other carrier materials. The charcoal was dried to a moisture level of 5% and grinded to a desired fineness preferably to pass a 100-200 mesh sieves and sterilized by autoclaving.
- Curing- The fresh *Rhizobium* broth culture was then blended manually with the finely powdered and sterilized carrier. The broth added was one third of the water holding capacity of the carrier. The mixture was then covered and left for curing for 2-10 days at 25-30ºC (Fig.3.3A-B).
- Packaging- After curing the carrier based inoculants were packed in pouches (each pouch measuring 10 x 8 cm). The pouches were labeled with proper strain names (Fig. 3.3.C). The pouch containing the carrier material with no inoculant served as a control. The pouches with the carrier based inoculants were then left for incubation at 30ºC during which the bacteria multiplied and reached to a required standard. The packets

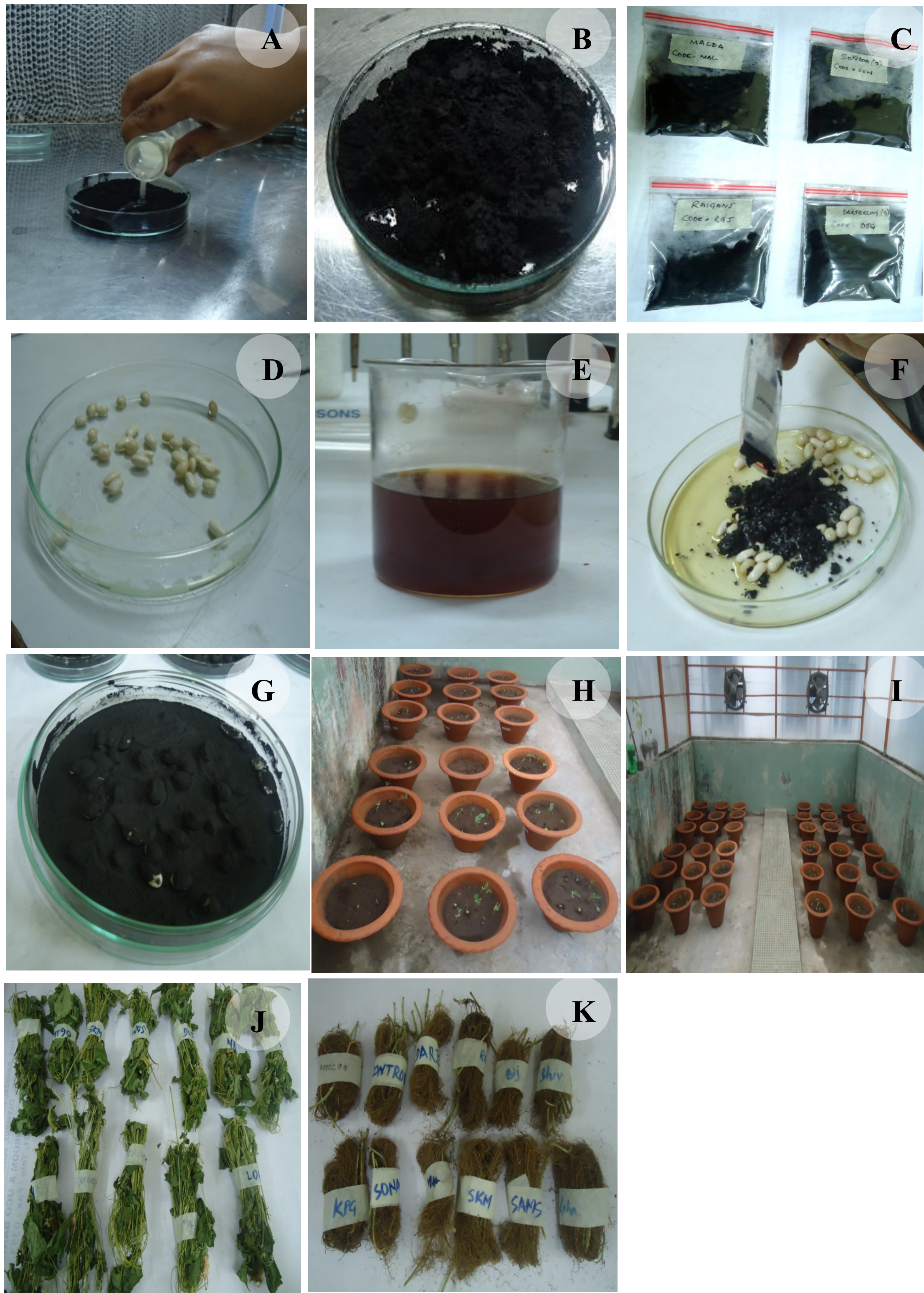

Fig 3.3: in vivo green house experiment.A: Pouring of *Rhizobium* inoculums in the carrier medium (charcoal); B: Curing of the carrier based inoculants; C: Pouches containing the carrier based inoculant; D: A plate with healthy seeds; E: A beaker containing 10% jaggery solution; F: Sprinkling of carrier based *Rhizobium* inoculants on the sticker coated seeds; G: Drying of the treated seeds; H-I:Sowing of the treated seeds in the earthen pots; J: Dried aerial part of the plants after harvest; K: Dried roots after harvest.

were then stored in a refrigerator (4ºC-15ºC) until further use (Tilak, 1991).

3.9.2.2. Seed inoculation with the carrier based Rhizobium inoculants

- Healthy French bean seeds were soaked in water for around 1 hr to remove the water soluble toxic substances.
- Bean seeds were surface-sterilized by washing with 96% ethanol for 30sec and 2.5% sodium hypochlorite for 3 min, and then rinsed several times with sterile, distilled water (Samavat *et al*., 2012) (Fig. 3.3.D).
- A 10% jaggery (gur) solution was prepared to use as a sticker for carrier based inoculant. About 10 ml of the cooled gur (sticker) solution was poured on the seeds, blended and coated uniformly and spread on a polyethylene sheet (Fig. 3.3E).
- Each contents of the carrier based inoculant packet were then sprinkled on the sticker coated seeds and mixed thoroughly (Fig. 3.3F).
- The seeds were then dried under shade for few hr followed by immediate seed sowing in the green house (Fig. 3.3G) (Tilak, 1991).

3.9.2.3. Green House Experiment

- The experiment was conducted in the Green house of Molecular Genetics Laboratory, Department of Botany, North Bengal University during growing season of *Phaseolus vulgaris* L. (December-February).
- The plants were allowed to grow under natural conditions of light, temperature and humidity. The day time temperature ranged from 22ºC to 27ºC and 17ºC to 19ºC during night.
- The green house experiment was carried out in a randomized block design with three replications to evaluate the selected rhizobial strain's effects on the common bean growth (Fig. 3.3H-I).
- A total of medium sized (10 inch diameter and 8 inch height) 39 earthen pots were used for the experiment.
- The soil of the University garden (Siliguri) was used for sowing of the seeds. The soil was meshed, sieved by passing through a 3 mm sieve and air dried.

- The analysis of different parameters of soil like the pH, organic carbon, nitrogen, potash and phosphate content were done and recorded.
- Around 5 kg of such soil was filled in autoclavable disposable plastic bags and sterilized at 15 lb psi for 1 hr.
- Each earthen pot were filled with around 5 kg of sterile soil and kept in the green house laid in a randomized block design with factorial arrangement of thirteen treatments replicated three times.
- In each pot 10 seeds coated with the carrier based *Rhizobium* inoculants were sown. Negative control consisted of the seed without rhizobial inoculant and the positive control consisted the seed treated with the reference *Rhizobium leguminosarum* MTCC 99
- After the emergence of seedlings, each pot were thinned to five seedlings. The pots were irrigated with sterile water as required. The French bean being a climber plant a support system had to be made with the help of ropes, wooden sticks and wires.

3.9.2.4. Post harvest analysis

- The pots were kept in a closed greenhouse in semi controlled conditions for two months, and the plants were harvested in the flowering phase after 40 - 45 days.
- The plants were uprooted carefully and the root system was washed to remove the soil particles.
- Growth promotion was evaluated in terms of increase in height, shoot and root length, and biomass in comparison to negative control.
- The drying of the root and the shoot system was done in a hot air oven at 70ºC for 3-4 days and the average dry weight per plant was calculated (Fig. 3.3 J-K) (Stajkovic *et al.* 2011).The dried shoot sample was then crushed, powdered and sieved for the nitrogen estimation by Kjeldahl method (Bremner *et al.* 1996) to calculate total N content in mg per plant.

3.9.2.5. Soil analysis

Analysis of different parameters of soil like pH, organic carbon (Walkley, 1947), nitrogen (Bremner *et al.* 1996), potash (Pratt, 1965) and phosphate (Truog, 1930) content were estimated after the harvesting of plants.

3.9.2.6. Data analysis

Means differing significantly were compared using Duncan's multiple range test (DMRT) at $p \leq 0.05$ with Statistica software version 5.0 (INC StatSoft, 1995). Correlation among different growth parameters of the plant were done by using bivariate correlation analysis using SPSS version 21(Pallant, 2013).

3.10. Diversity study through molecular characterization

The isolation and purification of genomic DNA of different *Rhizobium* strains was necessary before performing the different fingerprinting techniques namely RAPD, rep-PCR and PCR-RFLP for the diversity study.

3.10.1. Isolation of Genomic DNA from pure culture

The copious amount of exopolysaccharides produced by *Rhizobium* acts as a hindrance in the isolation of genomic DNA. Hence, to obtain a digestible chromosomal DNA, the CTAB/NaCl method was followed to isolate the *Rhizobium* genomic DNA using standard protocol of William and Feil (2012) with minor modifications.

- The bacterial strains were grown in YEM for 24-48 hr in OSI until the O.D. value reached 0.8 at 600nm.
- Thirty ml of 24-48 hr culture was put into a sterile oakridge tube and kept in the ice bucket for at least 1 hr. The tubes were centrifuged (REMI, Model No.C-24) at 5000Xg for 20 minutes at 4ºC.
- The supernatant was discarded and the pellet obtained was used for further isolation.
- The pellet was suspended in 5.67 ml 1X TE buffer (pH 8.0) (see appendix C for composition).
- Fifty five microliter (µl) of lysozyme (conc. 10mg/ml) was added to lyse the bacterial cell wall. The mixture was incubated for 5 min at room temperature.
- To the mixture 300 µl of 10% SDS was added and mixed well.
- Finally 50µl of proteinase K (20mg/ml) was added and blended well with gentle swirling.
- The mixture was then incubated in water bath (Rivotek, Cat# 50121002) for 1 hr at 37ºC. The tubes were mixed occasionally by gentle swirling in between the incubation period.
- Following incubation, 1ml of 5M NaCl was added to the solution and

mixed well. To that mixture, 0.8ml of hot (65ºC) CTAB/NaCl (see appendix C for composition) was added, mixed well and incubated at 65ºC for 10 min.

- The solution was extracted with equal volume of chloroform: isoamylalcohol (24:1) followed by gentle spinning at 5000Xg for 10min at room temperature. A white interface containing the debris could be visible after centrifugation. The clear upper aqueous layer was transferred to a fresh oakridge tube.
- Equal volume of phenol/ chloroform/ isoamyl alcohol (25:24:1) was added to the solutions, mixed well and centrifuged at 5000Xg for 20 min. The upper clear aqueous layer was transferred to a fresh oakridge tube.
- Approximately 0.6vol (8 ml) of chilled isopropanol stored at -20ºC was added to precipitate the nucleic acid. The tubes were shaken back and forth gently until a white cottony form of DNA was visible.

Thereafter, subsequently two steps were followed:

- In one step, the tubes were stored at –20ºC for 2hr. Thereafter, the tubes were spinned at 5000Xg for 30 min at 4ºC. The pellet thus obtained was washed with chilled 70% ethyl alcohol, air dried and dissolved in 500µl 1X TE buffer (pH 8.0).

However in the other step,

- The DNA was spooled out by hooking it onto the end of a micropipette that had been heat sealed and bend in a Bunsen flame. The spooled DNA was transferred to a fresh 2 ml Eppendorf tube containing cold 70% alcohol. The pellet was spinned at maximum speed for 5 min at room temperature, decanted and air dried.
- Finally the dried pellet was dissolved in 500µl 1X TE buffer (pH 8.0).

3.10.2. Purification of DNA

Major contaminants found in crude DNA like RNA and protein which may hinder in further downstream processing was eliminated in the purification procedure as follows:

3.10.2.1. RNase A treatment

RNase A (10mg/ml)(refer appendix C for composition) was dissolved in 500µl 1X TE buffer (pH 8.0) containing the crude DNA. The

mixture was incubated at 37ºC for 1 hr in a Dry water bath (GeNei^{TM} make, Cat#107173).

3.10.2.2. Protein clean up

To remove the protein impurities 500µl of phenol/chloroform/isoamylalcohol (25:24:1) kept as stock at 4ºC was added to the crude DNA. Micro centrifuge was done at 14000Xg for 2 min at room temperature. The aqueous phase was then transferred to a fresh microcentrifuge tube (Tarsons, Cat# 500010).

3.10.2.3. Ethanol precipitation of DNA

- The volume of the DNA was measured visually and 0.1 volume of 3M Sodium acetate (pH 5.2) was added and mixed well.
- It was followed by the addition of 2 volume of ice cold absolute ethyl alcohol and blended well by gentle swirling.
- The tubes were placed on ice at −20ºC for 30 mins and centrifuged at 14,000Xg for 10-15 min at 4 ºC.
- The DNA pellet was washed with 1 ml of chilled 70% ethanol, air dried and finally dissolved in 100 µl of 1X TE buffer (pH 8.0).

3.10.3. Quantification of DNA

The assessment of purity and concentration of the isolated crude DNA is necessary in molecular biology. The different techniques in molecular biology like the amplification of target DNA by Polymerase chain reaction (PCR) and the digestion of DNA by restriction enzymes (RFLP) etc. can be accomplished only if the quantification of DNA is done by a reliable method. Hence, both the spectrophotometric and agarose gel analysis were done to quantify the DNA.

3.10.3.1. Spectrophotometric measurement

- Spectrophotometer (Thermo UV1 spectrophotometer, Thermo Electron Corporation, England UK) was calibrated at 260nm and 280nm taking 600µl 1X TE buffer in a cuvette (Photon cell, New Jersey, USA).
- DNA (6µl diluted in 594µl of 1XTE) was taken in a cuvette and the optical density (OD) was recorded at both 260nm and 280nm.
- The DNA showing A_{260}/A_{280} ratio of around 1.8 was considered to be of good quality.
- The concentration (ng/µl) of isolated DNA was measured by

using the following formula:

$$DNA = \frac{OD_{260} X 50 XDF}{1000}$$

where DF stands for 'dilution factor'.

3.10.3.2. DNA analysis by gel electrophoresis

- A pure molecular biology grade, DNase free Agarose (0.8%, gelling temperature 36ºC) was used to cast the gel in 0.5X TBE (Tris-Borate-EDTA) buffer (refer Appendix C for composition) containing 7µl of Ethidium bromide (10mg/ml) on gel platform (100x70mm) (Tarsons, Cat # 7024).
- Five (5µl) of DNA samples were mixed with 3 µl of 6X gel loading dye (refer appendix for composition) and loaded in the wells carefully.
- Lambda DNA/ *Eco*RI/ *Hind*III double digest (2 µl) and 100 bp ladder were used as molecular markers to determine the size of genomic DNA.
- The gel was run at 50 volt (V) and 100 milliampere for 1.5 hr in the Midi Submarine Electrophoresis Unit (Tarsons, Cat #7050) connected to the Electrophoresis Power Supply Unit (Tarsons, Cat #7090).
- The gel was viewed on a UV Transilluminator (GeNei^{TM}, Cat # SF850). The molecular size of the genomic DNA were detected in the form of bands. The size of the bands were estimated with Photo-Capt Version 12.4, (Vilber Lourmat, USA).

3.10.4. Gel Photography

The gel was photographed by using an indigenously built Gel Documentation System fitted with Canon SLR camera (EOS 350D) bearing Marumi orange filter (58 mm YA2, Marumi, Japan). The software in usage for the purpose was EOS utility software.

3.10.5. DNA fingerprinting techniques

The DNA Fingerprinting techniques like RAPD, rep- PCR and 16SrDNA PCR-RFLP were done to study the genetic diversity of *Rhizobium* strains.

3.10.5.1. RAPD analysis

3.10.5.1.1. RAPD primers

A total of 37 oligonucleotide random primers (Chromous Biotech made) were screened for eighteen (18) different *Rhizobium* strains under study

Table 3.3. List of primers used for RAPD analysis

Primer ID	Sequence (5'→3')
OPA 01	CAGGCCCTTC
OPA 02	TGCCGAGCTG
OPA 03	AGTCAGCCAC
OPA 04	AATCGGGCTG
OPA 05	ATTTTGCTTG
OPA 06	GGTCCCTGAC
OPA 07	GAAACGGGTG
OPA 08	GTGACGTAGG
OPA 09	GGGTAACGCC
OPA 10	GTGATCGCAG
OPA 11	CAATCGCCGT
OPA 12	TCGGCGATAG
OPA 17	GACCGCTTGT
OPA 18	AGGTGACCGT
OPA 20	GTTGCGATCC
OPB 04	GGACTGGAGT
OPB 05	TGCGCCCTTC
OPB 06	TGCTCTGCCCC
OPB 07	GGTGACGCAG
OPB 08	GTCCACACGG
OPB 10	CTGCTGGGAC
OPC 19	GTTGCCAGCC
OPD 03	GTCGCCGTCA
OPF 09	CCAAGCTTCC
OPG 19	GTCAGGGCAA
OPH 04	GGAAGTCGCC
OPI 06	AAGGCGGCAG
OPI 14	TGACGGCGGT
OPN 04	GACCGACCCA
OPN 13	AGCGTCACTC
OPN 19	GTCCGTACTG
OPQ 01	GGGACGATGG
OPY 04	AAGGCTCGAC
OPY 07	GACCGTCTGT
DAF 4	CGGCAGCGCC
DAF 9	CCGACGCGGC
CRL 7	GCCCGCCGCC

(Table 3.3).

3.10.5.1.2. RAPD –PCR amplification

In a sterile 0.2 ml thin walled PCR tube (Tarsons,Cat#500050) following components were added for PCR reaction of 25 µl in the order as given below:

- Ready Mix TM Taq PCR Reaction Mix with $MgCl_2$ - 12.5 µl
- Primer- 1.25 µl
- Template DNA-2 µl (25 ng)
- Pyrogen free water- to a final volume of 25µl.

The ingredients were mixed evenly in a SpinWin PCR micro centrifuge (Tarsons, Cat# 1000).

- The PCR reactions were performed in Applied Biosystems, Thermal Cycler, 2720 PCR machine.
- The amplification cycle (modified protocol of Elboutahiri *et al*. 2009) set at 35 cycles were as follows:

Cycle 1: denaturation at 94ºC for 4 min, primer annealing at 36.0ºC for 1 min, primer extension at 72.0ºC for 2 min.

Cycle 2-34: denaturatin at 94ºC for 1 min, primer annealing at 36.0ºC for 1 min, primer extension at 72.0ºC for 2 mins.

Cycle 35:denaturatin at 94ºC for 1 min, primer annealing at 36.0ºC for 1 min, final extension at 72.0ºC for 7 min.

3.10.5.2. rep- PCR analysis

rep-PCR was performed for all *Rhizobium* strains under study to analyse the genetic diversity by

Table 3.4. List of primers used for rep-PCR analysis

rep-PCR	Primer ID	Sequence (5'→3')	References
REP-PCR	REP 1R-I REP2-I	CGGICTACIGCIGCIIII ICGICTTATCIGGCCTAC	de Bruijn(1992)
ERIC-PCR	ERIC 1R ERIC 2	CACTTAGGGGTCCTCGAATGTA AAGTAAGTGACTGGGGTGAGCG	de Bruijn(1992)
BOX-PCR	BOXA-1R	CTACGGCAAGGCGACGCTGACG	Versalovic *et al.*(1994)

exploring their repetitive sequences with the usage of specific repetitive element primers.

3.10.5.2.1. rep- PCR primers

The primers used for rep-PCR genomic fingerprinting were REP 1R-I and REP 2-I for REP-PCR , ERIC 1R and ERIC 2 for ERIC-PCR repetitive intergenic and BOX A 1R. The sequences of rep-PCR primers has been listed in Table 3.4.

3.10.5.2.2. REP- PCR amplification

The PCR reaction volume of 25 µl contained the following components:

- ReadyMix^{TM} Taq PCR Reaction Mix with $MgCl_2$ - 12.5 µl
- Primers:

  REP 1R-I -1.25 µl

  REP 2-I -1.25 µl
- Template DNA-2 µl
- Pyrogen free water- to a final volume of 25µl.
- The PCR were run for 35 cycles and the programme were set as follows:

Cycle 1:denaturation at 95ºC for 6 min, primer annealing at 40.0ºC for 1 min, primer extension at 65.0ºC for 8 min.

Cycle 2-34:denaturatin at 94ºC for 1 min, primer annealing at 40.0ºC for 1 min, primer extension at 65.0ºC for 8 min.

Cycle 35:denaturatin at 94ºC for 1 min,

primer annealing at 40.0ºC for 1 min,

and final extension at 65.0ºC for 16 min.

3.10.5.2.3. ERIC –PCR Amplification

The PCR mixture of the final volume 25µl contained the following components:

- ReadyMix ^{TM} Taq PCR Reaction Mix with MgCl2 - 12.5 µl
- Primers:

  ERIC 1R-1.25 µl

  ERIC 2R-1.25 µl
- Template DNA-2 µl
- Pyrogen free water- to a final

volume of 25µl

- The amplification cycle consisted of the following specifications:

Cycle 1:denaturation at 95ºC for 7 min, primer annealing at 52.0ºC for 1 min and primer extension at 65.0ºC for 8 min.

Cycle 2-34:denaturatin at 94ºC for 1 min, primer annealing at 52.0ºC for 1 min and primer extension at 65.0ºC for 8 min.

Cycle 35:denaturatin at 94ºC for 1 min, primer annealing at 52.0ºC for 1 min and final extension at 65.0ºC for 16 min.

3.10.5.2.4. BOX-PCR Amplification

The PCR mixture of the final volume 25µl contained the following components:

- Ready-Mix ™ Taq PCR Reaction Mix with $MgCl_2$ -12.5 µl
- Primers:

    BOX A 1R - 1.25 µl

- Template DNA-2 µl
- Pyrogen free water- to a final volume of 25µl
- The amplification cycle was same as ERIC-PCR.

3.10.5.2.5. RAPD and rep-PCR gel analysis

- All the PCR products of RAPD and rep-PCR after their respective amplifications were separated on 1.5% Agarose gel containing 7µl Ethidium bromide (10mg/ml) in 0.5X TBE buffer (pH 8.0).
- PCR product (12µl) was mixed with 4 µl of 6X gel loading dye (refer appendix C for composition), mixed well and then loaded in the agarose gel to run for 2.5 hr.
- Lambda DNA /*Eco*RI/ *Hind*III double digest (2 µl) and 100 bp ladder were used as molecular markers to determine the size of genomic DNA.
- The estimation of band sizes of the genomic DNA were done as mentioned in section 3.10.3.2 and the gel was photographed as mentioned in section 3.10.4.

The PCR were carried out thrice and only the clear and reproducible bands were compared with the adjacent marker DNA to estimate the sizes.

3.10.5.2.6. RAPD and rep-PCR Data analysis

- The RAPD and rep-PCR generated

fingerprints were scored in binary form i.e the presence of band as 1 and the absence of band as 0 and assembled in a data matrix.

- The datas were then analyzed with NTSYSpc (version 2.0) (Rohlf, 1998).
- A similarity matrix on the basis of band sharing was calculated using Dice coefficient (Nei and Li, 1979). UPGMA (Unweighted Pair Group Method with Arithmetic Mean), a simple agglomerative hierarchical method was used to construct a dendrogram.

3.10.5.3. *16SrRNA PCR-RFLP*

The 16SrDNA PCR products were subjected to restriction digestion with 8 different restriction endonucleases viz., *Alu*I, *Eco*RI, *Hae*III, *Hinf*I, *Hpa*II, *Mbo*1, *Taq*I and *Pst*I.

The lists of endonucleases (enzymes) with their cutting sites are given in Table 3.5.

The restriction digestion for each enzyme was carried out separately following the same procedure.

Each restriction digestion mixture of 20μl final volume contained the following components:

Table 3.5: List of restriction enzymes used for PCR-RFLP

Restriction Enzymes	Digestion Sites	Temp.
*Alu*I	AG↓CT	37ºC
*Eco*RI	G↓AATTC	37ºC
Haiti	GG↓CC	37ºC
Hinf I	G↓ANTC	37ºC
Hpa II	C↓CGG	37ºC
*Mbo*1	↓GATC	37ºC
Taq I	T↓CGA	65 ºC
Pst I	CTGCA↓G	37ºC

- Restriction enzyme buffer - 2 μl
- Restiction enzyme -0.5 μl
- PCR product - 5 μl
- BSA -0.2 μl

Pyrogen free water - to make up the final volume

The mix was incubated at specific temperature(please refer table 3.5) for different enzymes in a dry bath (Genei ™ make, Cat# 107173) for 1 hr.

The total restriction digested products were loaded and separated on 2% (w/v) agarose gel solution and run in 0.5X TBE buffer (pH 8.0) at 50 volt and 100 amp for 2 hr 30 min.

Lambda DNA/*Eco*RI /*Hind* III double digest (2 μl) and 100 bp ladder were used as molecular markers.

The band sizes of genomic DNA were estimated as mentioned in section

3.10.3.2 and the gel was photographed as mentioned in section 3.10.4.

3.10.5.3.1. 16SrDNA PCR-RFLP Data analysis

The binary data scored as 0 and 1 were analyzed with NTSYSpc2 (Rohlf, 1998). A similarity matrix was calculated using Dice coefficient (Nei and Li, 1979).

3.11. Exploration of the partial 16SrDNA genome of *Rhizobium* strains

3.11.1. 16S rDNA –PCR

The purified DNA whose quality assessment by the spectrophotometer was found to be 1.8, was deemed satisfactory for the 16SrDNA PCR amplification. The PCR was performed to amplify a partial region of the conserved gene 16SrRNA.

3.11.1.1. Primers used for 16SrDNA-PCR

A primer pair consisting of

337F–5’GACTCCTACGGGAGGCWGCAG3’ &

907R–5’CCGTCAATTCCTTTRAGTTT-3’

was used to amplify the partial 16SrDNA region of the genome.

3.11.1.2.PCR amplification of 16S rDNA region

Each PCR tube with a final volume of 25µl contained the following:

- Go Taq Green Master Mix -12.5 µl
- Primers :

  337F -1.25 µl

  907 R -1.25 µl
- Template DNA – 2 µl
- Pyrogen free water - to a final volume of 25µl

The PCR were run for 35 cycles and the programme specifications were as follows:

Cycle1: denaturation at 95ºC for 5 min, primer annealing at 54ºC for 45s and primer extension at 72ºC for 2 min.

Cycle 2-34: denaturation at 95ºC for 45s, primer annealing at 54ºC for 45s and primer extension at 72ºC for 2 min.

Cycle 35: denaturation at 95ºC for 45s, primer annealing at 54ºC for 45s and primer extension at 72ºC for 7 min.

- The amplified PCR products were separated on 1.5% (w/v) agarose gel and run in 0.5X TBE buffer (pH 8.0) at 50 volt, 100 amp for 2 hr 30 min.

- Lambda DNA/*Eco*RI /*Hind*III double digest (2 µl) and 100 bp ladder were used as molecular markers.
- The band sizes of genomic DNA were estimated as mentioned in section 3.10.3.2 and the gel was photographed as mentioned in section 3.10.4.

3.11.2. Sequencing of partial region of 16SrDNA

To confirm the identity of PCR bands generated by 16SrDNA primer pair, the corresponding amplification products were directly sent for sequencing.

A total of 50 µl 16SrDNA PCR product was taken in each PCR tube, labeled properly, sealed with parafilm, packed in an airtight box with icepacks and sent for sequencing to Chromous Biotech Pvt. Ltd., Bangalore-5600092 (www.chromous.com).

3.11.3. Sequence analysis

The raw DNA sequence data samples received from Chromous Biotech, Bangalore were individually compared to those from GenBank using Basic

	Sequence type	Description	Info	Select
	rRNA gene	For ribosomal RNA genes from prokaryotic, nuclear or organellar DNA. All rRNAs are considered partial.	ⓘ	Select
	Single CDS genomic DNA	For complete or partial coding sequence (CDS) derived from genomic DNA. This checklist will not accept segmented genes (i.e., with intron regions) so should be used for prokaryotic, organellar genes or for submitting a single exon.	ⓘ	Select
	Single CDS mRNA	For complete or partial single coding sequence (CDS) derived from mRNA (via cDNA). Do NOT use for submission of VIRTUAL transcripts (TSA or Unigene clusters) - use TSA CDS Annotated checklist.	ⓘ	Select
NEW	Multi-Exon Gene	For the submission of single complete or partial multi-exon genes from in vivo genomic DNA. This checklist captures the gene region but does not capture exon, intron or CDS features. No translation will be generated. If precise annotation or translation is required, please use an alternative submission route. Single exon genes or sequences covering a single exon only from a multi-exon gene can be submitted using the Single CDS Genomic DNA checklist. For HLA/MHC genes which cannot be submitted using 'MHC gene 1 exon' or 'MHC gene 2 exon' checklist, please use the Entry Upload option (see: http://www.ebi.ac.uk/ena/submit/entry-upload-templates)	ⓘ	Select
	MHC gene 1 exon	For partial MHC class I or II antigens containing one exon ONLY.	ⓘ	Select
	MHC gene 2 exons	For partial MHC class I or II antigens containing two exons ONLY. An intron feature should only be used when the intron region has actually been sequenced. If the intron has not been sequenced, or only partially sequenced, please fill the non-sequenced gap with 100 Ns.	ⓘ	Select
NEW	ncRNA	For non-coding RNA (ncRNA) transcripts or single-exon genes of prokaryotic or eukaryotic origin with the exception of the ribosomal RNA (rRNA) and transfer RNA (tRNA.)	ⓘ	Select
	Satellite DNA	For submission of Satellites, Microsatellites and Minisatellites. Complete or partial single polymorphic locus present in nuclear and organellar DNA that consists of short sequences repeated in tandem arrays	ⓘ	Select
NEW	Mobile Element	For the submission of a single complete or partial mobile element. This checklist captures the mobile element feature but does not allow for granular annotation of component parts, such as coding regions, repeat regions and miscellaneous features within the mobile element itself. If precise annotation or translation is required, please use an alternative submission route.	ⓘ	Select
	Gene Promoter	For submission of uni- or bi-directional gene promoter regions. Please note that CDS is not annotated; if you wish to include the start of the coding region(s), please leave a comment with the coordinates of the start site(s)	ⓘ	Select

Submission type	Description	Info	Select
Entry upload	Upload pre-prepared EMBL format files.	-	Select

Figure3.4: EMBL format used for submission of 16SrRNA sequences of *Rhizobium* isolated from root nodules of *Phaseolus vulgaris*.

Table 3.6: Details of partial16SrRNA sequences retrieved from NCBI for phylogenetic analysis

Family	Strain names	GenBank Accn. #	References
Rhizobiaceae (*Rhizobium/ Agrobacterium* group)	*R. leguminosarum* ATCC 14480	AY509900	Ramirez-Bahena *et al*., 2008
	R. leguminosarum bv. *viciae* USDA 2370	NR_118339	Faghire *et al*., 2012
	R. phaseoli ATCC 14482	EF141340	Ramirez-Bahena *et al*., 2008
	R. phaseoli NBRC 14785	AB680664	Nakagawa *et al*., 2011*
	R. tropici H 52	EU488756	Dall' Agnol *et al*., 2014
	R. tropici CIAT 899	EU488752	Ribeiro *et al*., 2009
	R. etli 1002	JQ670251	Shamseldin *et al*., 2014
	R. etli 975	JQ670250	Shamseldin *et al*., 2014
	R. gallicum R602sp	NR_036785	Amarger *et al*., 1997
	R. gallicum CbS-18	AF008128	Sessitsch *et al*., 1997
	R. giardinii ASN-6	JQ946072	Vicente *et al*.,2012*
	R. giardinii H152	EU488750	Ribeiro *et al*., 2009
	R. soli DS-42	NR_115996	Yoon *et al*., 2010
	R. vignae CCBAU 05176	NR_117440	Chen *et al*., 2011
	R. leucaenae CCGE 522	JF318175	Ribeiro *et al*., 2012
	R. pisi DSM 30132	NR_115253	Ramirez-Bahena *et al*., 2008
	*Allorhizobium*sp. G9.8	LN845909	Pulawska, 2015*
	A. undicola	Y17047	de Lajudie *et al*., 1998
	Neorhizobium galegae G323	KT869545	Bromfield and Cloutier, 2015*
	N. galegae G1	KT869496	Bromfield and Cloutier, 2015*
	Agrobacterium sp. EECC-441	KP860648	Mulugeta *et al*., 2015*
	A. tumefaciens W230	GU826585	Bruck *et al*., 2012
	Shinella sp. CTN-12	FJ598324	Liang *et al*., 2011
	S. granuli Ch06 (KCTC 12237)	AB187585	An *et al*., 2006
	Rhizobium sp (Rh S-3)	LN823958	Rai and Sen, 2015*
	Rhizobium sp (Rh K-1)	LN823959	Rai and Sen, 2015*
	Rhizobium sp (Rh NBU-8)	LN823960	Rai and Sen, 2015*
	Rhizobium sp (Rh KPG-5N)	LN823961	Rai and Sen, 2015*
	Rhizobium sp (Rh SAM-12)	LN823962	Rai and Sen, 2015*
	Rhizobium sp (Rh SHV-1)	LN823963	Rai and Sen, 2015*
	Rhizobium sp (Rh MIR-6)	LN823964	Rai and Sen, 2015*
	Rhizobium sp (Rh MAL)	LN 833880	Rai and Sen, 2015*
	Rhizobium sp (Rh SKM)	LN 833881	Rai and Sen, 2015*
	Rhizobium sp (Rh RGJ)	LN 833882	Rai and Sen, 2015*
	Rhizobium sp (Rh CBR)	LN 833883	Rai and Sen, 2015*
Sinorhizobium/ Ensifer group	*Ensifer mexicanus* CAF249	FJ405371	Peng and Jiao, 2008*
	E. adhaerens ORS 529	AM946571	Merabet *et al*., 2010
	Sinorhizobium meliloti CCBAU 83884	EU379948	He *et al*., 2011
	S. americanum CFNEI 156	NR_02525	Toledo *et al*., 2003
Bradyrhizobiaceae	*Bradyrhizobium japonicum* LMG 6138	NR_118981	Willems and Collins, 1992
	B. elkanii USDA 76	NR_117947	Sanchez-Canizares *et al*., 2011
Phyllobacteriaceae	*Mesorhizobium ciceri* CB8	JX891459	Alavian *et al*., 2012*
	M. albiziae CCBAU 61158	NR_043549	Wang *et al*., 2007
Xanthobacteriaceae	*Azorhizobium caulinodans* LMG 6465	NR_119216	Rainey and Wiegel, 1996
	A. doebereinerae BR5401	NR_041839	de Souza *et al*., 2006
Rhodobacteriaceae	*Rhodobacter sphaeroides* CNT-2A	FR731160	Sanjukta *et al*., 2011*

*Not published in any journal

Local Alignment Search Tool (BLASTN) programme (Altschul *et al*. 1990) under NCBI (National Centre for Biotechnology Information) website (http://blast.ncbi.nlm.nih.gov/ Blast.cgi).

3.11.4. Sequence submission in public domain

The raw partial 16S rDNA sequences of *Rhizobium* were submitted online to European Molecular Biology Laboratory (EMBL) nucleotide sequence database (http:// www.ebi.ac.uk/embl) with proper annotations and descriptions after registering to the website (please refer Fig. 3.4). The necessary guidelines were followed during the submission process and the sequences were submitted in the FASTA format.

3.11.5. Phylogenetic analysis based on 16SrRNA gene

A phylogenetic tree was constructed comparing the eleven partial 16SrDNA sequences with that of the sequences of 35 different standard rhizobial strains obtained from gene bank (http:// www.ncbi.nlm.nih.gov). The standard reference strains falling under the order Rhizobiales downloaded from NCBI consisted of 29 strains from the family Rhizobiaceae, 2 strains from Bradyrhizobiaceae, 2 from Phyllobacteriaceae and 2 from Xanthobacteriaceae. The details of description of the strains and their gene bank accession numbers has been given in details in Table 3.6.

A phylogenetic tree was constructed by RAxML (Randomnized Axelerated Maximum Likelihood, version 8) with 1000 bootstrap value using the PROTCAT_GAMMAI WAG substitution model.

Rhodobacter sphaeroides of Rhodobacteriales was used as an out group for 16SrDNA phylogenies.

3.12. Whole genome sequencing

The draft genome of *Rhizobium* strain RSm-3 was generated at the Hubbard Genome Center (University of New Hampshire, Durham, NH) using Illumina technology (Kuhn *et al.* 2004). The library was generated by the dilute Nextra method as per the manufacturer's instructions (Illumina Inc., San Diego, CA). The libraries were sequenced using paired end reads with a length of 150 base-pairs using the Illumina HiSeq2000 by the Hubbard Center for Genome Studies (UNH, Durham, NH). Trimmomatic was used to remove extra adapters and to filter the quality of raw reads

(Bolger *et a*l. 2014) . SPAdes Version 3.6.0 (Bankevich *et al*. 2012) and Alopaths were used to assemble the genome. The raw DNA sequence data of the whole genome was compared to those from GenBank using BLASTN programme (Altschul *et al*. 1990) under NCBI. Genome annotation was performed with the IMG (Integrated Microbial Genomes) (Markowitz *et al*. 2013).

3.13. Bioinformatics study

3.13.1. Retrieval of sequences:

Five strains of *Rhizobium: R. leguminosarum* bv. *viciae* 3841, *R. leguminosarum* bv. *trifolii* WSM1325, *R. etli* bv. *phaseoli* IE4803 and *R. etli* bv. *mimosae* Mim1 along with our own newly sequenced *Rhizobium* sp. RSm-3 were considered for the bioinformatics study.

Gene sequences along with their respective amino acid sequences were obtained from IMG database (www.img.jgi.doe.gov) (Markowitz *et al.* 2012) for first four genomes while for RSm-3, gene sequences and translated protein sequences were determined by the annotation package RAST(rast.nmpdr.org), an automatic annotation server for microbial genomes. Genes involved in the pathways of nitrogen fixation, siderophore formation and Indole Acetic Acid (IAA) formation were screened and retrieved from KEGG (Kyoto Encyclopedia of Genes and Genomes) database. These sets of genes were considered for codon usage analysis. Normally, the ribosomal protein genes are considered to be the most stable and highly expressed genes in a genome, hence they were taken as reference set for the analysis of codon adaptation index (CAI).

3.13.2. Codon Usage variation Analysis

The codon usage disparity in various *Rhizobium* genomes have been investigated by several potent indices like number of Guanine and Cytosine in the nucleotide sequence (G+C content), frequency of G and C in the third position of codons (GC3s), effective number of codons (Nc) (Wright 1990) and frequency of optimal codon (Fop) (Ikemura, 1985). All these parameters were calculated by CodonW (Ver.1.4.2) package (Peden, 2000). These parameters are crucial for determining the extent of codon usage bias in concerned genomes and highlight the factors affecting codon usage pattern.

The effective number of codons (Nc) is a parameter that can measure codon bias of synonymous codons. It is a quantitative measure reflecting the frequency of a small subset of codons used by a gene (Wright, 1990) and its value ranges from 20 (on usage of one codon per amino acid) to 61 (on usage of all the codons with equal frequency). Nc is computed as in equation (1)

$$Nc = 2 + S + \{29/[S^2 + (1-S)^2]\} \quad (1)$$

where, S represents GC3 values, Frequency of optimal codon (Fop) represents the fraction of synonymous codons that are optimal codons (Ikemura 1985). Its value ranges from 0 (meaning a gene has no optimal codons) to 1 (when a gene is exclusively comprised of optimal codons). Fop is generally determined by the equation 2(a):

$$Fop = N_{oc} / N_{sc} \quad (2a)$$

The original equation is modified in equation (2b), when rare codons are identified, as

$$Fop_{(mod)} = N_{oc} - N_{rc} / N_{sc} \quad (2b)$$

Where N represents the frequency of each codon type used, whereas N_{oc}, N_{rc} and N_{sc} stands for optimal codons, rare codons and synonymous codons respectively.

The codon bias index (CBI) is another important estimator of directional bias and determines the level to which a gene uses a subset of optimal codons. The codon bias index value ranges from 0 to 1.It is calculated by equation (3) as follows:

$$CBI = N_{opt} - N_{ran} / N_{tot} - N_{ran} \quad (3)$$

where N_{opt}= number of optimal codons; N_{tot} = total number of synonymous codons; N_{ran}= expected number of optimal codons in cases where codons are assigned randomly.

3.13.3. *Expression prediction*

The codon adaptation index (CAI) is a commonly used measure of codon usage within a gene relative to a reference set of genes (usually ribosomal protein genes). The CAI values were computed using the web-based application the CAI Calculator2 (http://userpages.umbc.edu/~wug1/codon/cai/cais.php) (Wu *et al.* 2005). The CAI value varies between 0 and 1·0 (Sharp and Li, 1987), with higher CAI values indicating that the gene of interest has a codon usage pattern more similar to that of the reference genes. CAI is usually calculated based on the following equation:

$$CAI = \exp\left(\frac{1}{L}\sum_{k=1}^{L}\ln \omega k\right) \quad (4)$$

where, ω_K signifies the relative adaptivity of the Kth codon and L represents the number of synonymous codons in the gene.

Ideally, the reference set in CAI is composed of highly expressed genes, so that CAI provides an indication of gene expression level under the assumption that there is translational selection to optimize gene sequences according to their expression levels. Generally ribosomal proteins are highly expressed in a given genome and as a consequence are taken as the reference set for calculation of CAI values.

3.13.4. Relative synonymous codon usage

Relative synonymous codon usage is a simple measure of the heterogeneity in the usage pattern of synonymous codons (Sharp and Li, 1987). RSCU values represent the number of times a particular set of codon is observed with respect to the number of times it would have been expected in case of a uniform synonymous codon usage. RSCU is formulated as:

$$RSCU = \frac{Freq_{obs}}{Freq_{exp}}$$

where, $Freq_{obs}$ is observed frequency of a codon and $Freq_{exp}$ is the expected frequency of the same codon (if all the codons were used equally)

RSCU value greater than one means that the observed frequency of synonymous codon is more compared to the expected frequency, while RSCU value lower than one indicates the opposite (dos Reis *et al*. 2003).

3.13.5. Strand biased property of Rhizobium genomes

Information regarding the strand bias of *Rhizobium* genomes was obtained from IMG database.

3.13.6. *Cluster of Orthologs groups (COG)*

Information regarding the COG groups of genes in *Rhizobium* was gathered from IMG database.

3.13.7. Statistical analysis

All the statistical analysis was done via SPSS software version 2.6. ANOVA and Two tailed t Tests were done with correlation coefficient at 0.01 and 0.05 level where applicable.

3.13.8. *Comparative genomic study among selected Rhizobium strains*

Blast matrix, pan-core genome

analysis, circular genome view were considered for comparative genome analysis.

3.13.9. Blast matrix generation

The blast matrix is a visual presentation of proteome comparison using BLAST. The targeted sequences are compared to each other and a BLAST hit is considered to be significant when 50% of the alignment is found to be identical. In Blast matrix, protein families are built through single linkage.

3.13.10. Pan- Core plot analysis

This is a bit different use of BLAST for comparing different proteomes using the 50/50 cut-off. The pan genome is the additive protein families of all genomes under consideration whereas the core genome consists of only those families which are being shared by all considered genomes. Hence, with addition of more and more genomes, the number of pan genome increase whereas, the core genome family decreases. To construct the pan-core genome plot, at first a machine readable matrix was created from the protein sequence and then, the pan-core plot was generated by a program called pan-core plot executed in CMG Biotools.