Chapter 4
Results and Discussion

4.1. Collection of germplasm

Root nodules of French bean (*Phaseolus vulgaris* L.) were collected as germplasm from different geographical locations of North Bengal and Sikkim which has been listed in table 3.1 (in Materials and Methods section). The germplasm was collected during the month of January - February in plain region and July-August in the hilly region from the cultivated land.

The length of the roots varied from 30 to 60 cm, but the main root volume was found to be within the upper 20cm of the soil. The nodules were found to be distributed on the lateral roots of the upper and middle parts of the root system.

Developing nodules were whitish yellow in color which turned light brownish upon storage. Crespi and Galvez (2000) have grouped the nodules into two different types, the determinate and the indeterminate type. Determinate nodules are usually formed on tropical and subtropical legume plants while the indeterminate nodules are found on root of temperate legumes. The former type has limited meristematic growth and the nodules are often approximately spherical in shape, while the latter type show indeterminate growth, have prolonged apical meristematic growth and are often cylindrical in shape (Sprent, 2009).

The French bean nodule represented the former type in which the nodule growth takes place by cell expansion rather than by cell division. The mature nodules were spherical in shape and approximately 2-5mm in diameter (Fig. 4.1). It was found that both the freshly collected as well as the carefully preserved nodules (at –20ºC) gave good results.

4.2. Isolation of *Rhizobium*

A total of twenty eight strains of *Rhizobium* were isolated from the root nodules following the protocol of Bedi and Naglot (2011). For the successful isolation of *Rhizobium,* the surface sterilization of root nodules with the H_2O_2 treatment was found to be

Fig. 4.1. Root of *Phaseolus vulgaris* with mature nodules

effective in eliminating the other soil microorganisms.

Distinct whitish to light pinkish colored *Rhizobium* colonies appeared in the YEMA medium amended with 0.0025% concentration of Congo Red dye after 24-48 hr incubation as per the procedure described in 3.3.1(Fig. 4.2). Congo red (diphenyldiazo-bis-α naphthyl amine sulfonate) is frequently included in culture media for isolating *Rhizobium* sp. or for testing the purity of *Rhizobium* cultures (Kneen and LaRue, 1983). According to Jordan and Allen (1974) rhizobia produces white color or absorb the dye weakly, whereas many other bacteria including *Agrobacterium* sp. take up the dye strongly. Similarly, the colonies of the bacterial strains under study did not absorb the Congo red dye confirming the absence of contaminants like *Agrobacterium* in the culture. The colonies of *Rhizobium* strains under study appeared to be similar to the colonies of reference strain *R. leguminosarum* MTCC-99. The strains were then obtained in pure colony form by repeatedly streaking on the YEM plates. The maintenance of cultures in YEM slants at 4°C helped in the post isolation workup.

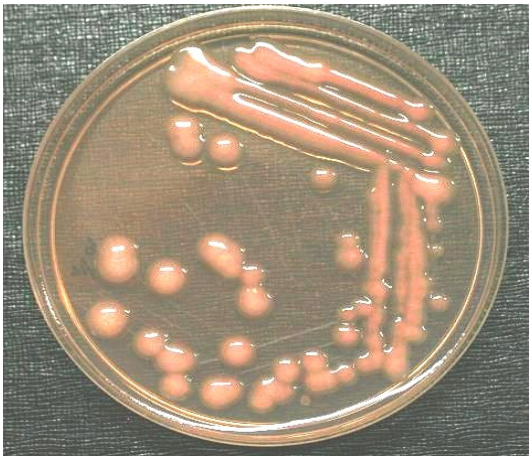

Fig. 4.2. *Rhizobium* colonies in YEM+Congo red medium

4.3. Plant infectivity test

Infection test of *Phaseolus vulgaris* seedlings grown in the improvised Leonard jars (Fig.4.3A-B) showed the appearance of healthy nodules after 30-45 days (Fig. 4.3C-D). They were light brown in color and 1-1.5 mm in diameter.

The modified nitrogen free Jensen's medium in the lower half of the

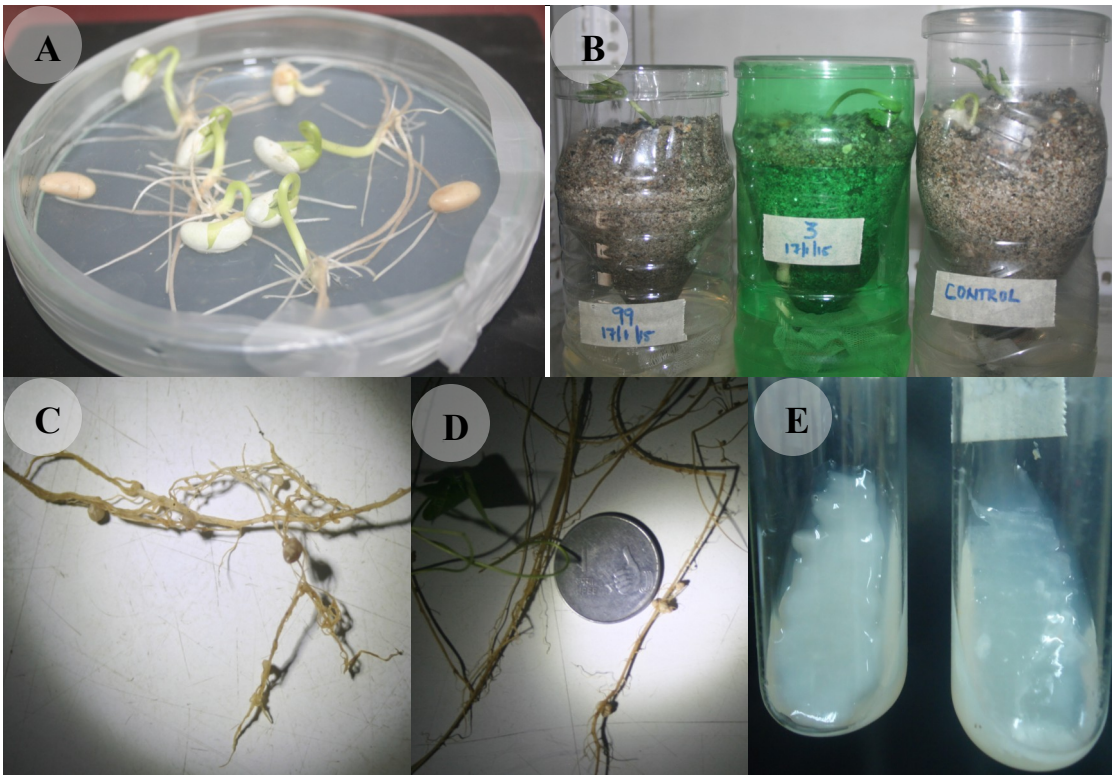

Fig. 4.3 (A) Germination of French bean seeds in water agar medium; (B) French bean seedlings grown on the sterilized sand in the improvised Leonard jar Assembly; (C & D) Formation of nodules by Koch's postulation; (E) Subculturing and maintenance of pure colony of *Rhizobium* in a slant.

Leonard jar supplied the nutrient required for the growth of seedlings while the need of nitrogen was fulfilled by the inoculated symbiont as expected.

The presence of appropriate rhizobia in the soil is a critical and a primary factor for the nodule formation (Pepper *et al*. 1991). Hence, in this present study, seventeen out of 28 strains could infect their host plant and produce nodules, ensuring that these 17 isolates are indeed *Rhizobium.* The sterile condition of the whole experimental setup with constant watering using sterile water fetched no chances of contamination by other soil microorganisms.

In this experiment, *R. leguminosarum* MTCC 99 was used as positive control which was found to be successful in infecting and nodulating the plant. On the other hand the negative control showed stunted growth in comparison to inoculated seedlings and at no stage of the experiment was nodules found in them.

The authenticated pure strains were then cultured on YEM plates and further subcultured and maintained in slants at an interval of every one month (Fig. 4.3E). The confirmed

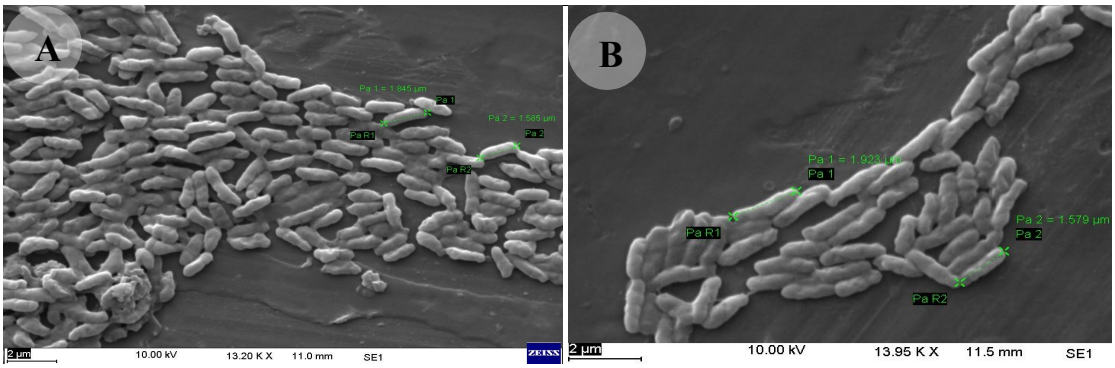

Fig. 4.4 (A-B) Scanning electron microscopic images of *Rhizobium* cells examined under Carl Zeiss Scanning Electron Microscope

seventeen strains were further subjected to various morphological, biochemical, physiological and molecular characterization.

4. 4. Microscopy

4.4.1. Light microscopy

The bacterial cells were found to be gram negative, single and rod shaped. The size of the cells varied from 1.5-2.5 µm.

4.4.2. Scanning Electron microscopy

The SEM images of the pure cultures of RMa-13, RSm-3, RSv-1, SKM-N and MTCC-99 from an exponential phase revealed the bacterium to be rod shaped and non-motile. The lengths of the cells were found to vary between 1.442-2.395 µm (Fig.4.4A-B). Not much work has been done on the SEM of *Rhizobium* pure culture from root nodules of *P. vulgaris*. Kesari *et al*. (2013) revealed the *Rhizobium pongamiae* sp. nov. from root nodules of *Pongamia pinnata* to show similar kind of structure under SEM. The dimension of the cell recorded was of 0.4-0.5 μm width and 1.4–1.6 μm length.

4.5. Morphological characterization

4.5.1. Colony morphology

Majority of the colonies of isolated strains were found to be circular with entire margin except RSv-1 and BIJ which were found to be oval in shape (Fig. 4.5). All the colonies were found to be convex, semi-translucent, raised,

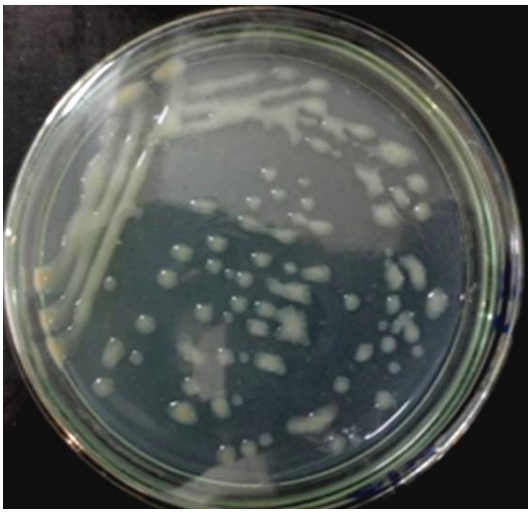

Fig. 4.5. Pure *Rhizobium* colonies on YEM plates

Table 4.1. Morphological characters of rhizobial isolates collected from various regions of North Bengal and Sikkim.grown

Isolates	Microscopic observation			Colony morphology					Colony size (mm) After	
	Sh	Arr	GS	For	Mar	El	Text	De	48 hr	72 hr
MTCC99	Rd	S	-ve	C	E	R	Muc	ST	1.13±0.06	1.94±0.04
K-1	Rd	S	-ve	C	E	R	Muc	ST	1.33±0.03	2.0 ±0.09
DJG	Rd	S	-ve	C	E	R	Muc	ST	1.70±0.12	3.98±0.06
RSm-3	Rd	S	-ve	C	E	R	Muc	ST	1.43±0.06	2.82±0.03
KPG-5N	Rd	S	-ve	C	E	R	Muc	ST	1.20±0.09	2.64±0.12
MIR-6	Rd	S	-ve	C	E	R	Muc	ST	1.33±0.03	2.86±0.03
SKM-N	Rd	S	-ve	C	E	R	Muc	ST	1.47±0.12	3.33±0.06
SKM-W	Rd	S	-ve	C	E	R	Muc	ST	1.57±0.06	3.78±0.12
SKM-E	Rd	S	-ve	C	E	R	Muc	ST	1.40±0.12	3.08±0.06
NBU-8	Rd	S	-ve	C	E	R	Muc	ST	1.37±0.03	3.12±0.06
RSv-1	Rd	S	-ve	O	E	R	Muc	ST	1.23±0.03	3.15±0.03
BLG	Rd	S	-ve	C	E	R	Muc	ST	1.23±0.12	3.21±0.06
SAM-12	Rd	S	-ve	C	E	R	Muc	ST	1.40±0.09	3.88±0.12
CBR	Rd	S	-ve	C	E	R	Muc	ST	1.33±0.06	3.49±0.06
BIJ	Rd	S	-ve	O	E	R	Muc	ST	1.40±0.06	3.51±0.03
JPG	Rd	S	-ve	O	E	R	Muc	ST	1.30±0.06	3.44±0.06
RMa-13	Rd	S	-ve	C	E	R	Muc	ST	1.20±0.03	2.98±0.06
RGJ	Rd	S	-ve	C	E	R	Muc	ST	1.13±0.03	2.00±0.06

Sh= Shape; Arr= Arrangement; GS= Gram staining; For= Form; Mar= Margin; El= Elevation; Text= Texture; De= Density; Rd= Rod; S= Single; -ve = Negative; C= Circular; O= Oval; E= Entire; R= Raised; Muc= Mucous; ST=Semi Translucent

and mucilagenous in nature. The mucosity was due to the production of copious extracellular polysaccharide slime (Jordan, 1984). The diameter of the typical colonies was found to be 1.13-1.70 mm after 48 h and 1.94-3.98 mm after 72 h of growth. The colonies were found to be light pink in color, a character shared with species of *Bradyrhizobium* due to the inability to absorb Congo red from yeast extract mannitol agar medium. The details of the colony morphology have been depicted in Table 4.1**.**

All the isolates appeared to be fast growers as the growth could be seen within 2-3 days of incubation. All these results concur with the observation made by various workers (Jordan, 1984; Deora and Singhal, 2010; Bedi and Naglot, 2011; Deshwal and Chaubey, 2014)**.**

4.5.2. Gram staining

The microscopic examination of the *Rhizobium* strains subjected to Gram staining revealed the bacteria to be gram negative, single and rod shaped (Table 4.1).

4.6. Biochemical characterization

The details of the observation of different biochemical tests in the form of presence or absence of growth, color change etc has been depicted in Fig. 4.6 and table 4.2.

Table 4.2: Results of biochemical tests performed to characterize rhizobial isolates of various regions of North Bengal and Sikkim

Isolates	HT	KT	AAPT			GPA	MB	SH	CPT	GL	TSI	CT	UT	NRT
			YBT	Cc	pH									
MTCC-99	-	-	+++	Y	6.5	++	+++	-	-	-	-	-	+	+
K-1	+	-	+++	Y	6.2	+	+++	-	-	-	-	-	+	+
DJG	-	-	++	Y	6.5	-	++	-	+	-	-	-	+	+
RSm-3	+	-	+++	Y	6.9	++	+++	+	-	-	-	+	+	+
KPG-5N	-	-	++	Y	6.3	-	++	-	-	-	-	-	+	+
MIR-6	-	-	++	Y	6.1	-	++	-	-	-	RR	-	+	-
SKM-N	-	-	+++	Y	6.3	-	+	-	-	-	RY	-	+	+
SKM-W	-	-	++	Y	6.5	-	+++	-	-	-	RY	-	+	+
SKM-E	-	-	+++	Y	6.2	-	+++	-	-	-	RY	-	+	+
NBU-8	-	-	+++	Y	6.1	-	+++	-	-	-	RY	-	+	+
RSv-1	-	-	++	Y	6.2	-	+++	+	-	-	RY	+	+	+
BLG	-	-	+++	Y	6.1	+	+++	-	-	-	-	-	+	+
SAM-12	-	-	+++	Y	6.2	++	+++	-	+	-	-	-	+	+
CBR	+	-	+++	Y	6.1	++	+++	-	++	-	-	-	+	+
BIJ	-	-	+++	Y	5.8	++	+++	-	++	-	RY	-	+	+
JPG	-	-	++	Y	5.9	++	+++	-	+	-	-	-	+	-
RMa-13	-	-	+++	Y	5.9	-	+++	+	-	-	RY	+	+	+
RGJ	-	-	+++	Y	6.4	-	+++	-	-	-	RY	-	+	+

HT-Hofer's test; KT-Ketolactase test; AAPT-Acid alkaline production test; GPA-Glucose peptone agar; MB-Methylene blue; SH-Starch hydrolysis; CPT-Cellulase production test; GL-Gelatinase liquefaction; TSI-Triple sugar iron agar test; UT-urease test; NRT-Nitrate reductase test

4.6.1. Hofer's alkaline medium

The *Rhizobium* strains were subjected to Hofer's alkaline (pH 11) plates to check the purity of the bacteria, mainly to identify the contaminant like *Agrobacterium* (Hofer, 1935; Allen and Allen, 1958).

Except few isolates like RSm-3, CBR and K-1, all the *Rhizobium* strains did not grow in Hofer's alkaline medium. Similar result was also observed by (Deshwal and Chaubey, 2014) while working with *Rhizobium* isolates isolated from the root nodules of *Pisum sativum* (L.).

4.6.2. Ketolactase test

Ketolactase is an enzyme which converts lactose to ketolactose which is

detected by Benedict's reagent.

In this present study, when the *Rhizobium* strains were grown on lactose agar medium, no yellow zone was observed around the colonies after flooding with Benedict's reagent. This may be due to the absence of production of 3-ketoglycosides (Bernaerts and De Ley, 1963). Absence of yellowish zones of Cu_2O around all the *Rhizobium* colonies indicated the purity of the isolates (Bernaerts and De Ley, 1963; Deshwal and Chaubey, 2014).

4.6.3. Acid alkaline production test

All the studied strains inoculated on YEMA with Bromothymol blue (BTB) changed the color of the medium to yellow showing the production of acid (Fig. 4.6A). The yellow color appeared due to reaction with BTB which was supplemented in YEMA. The strains are considered to be fast growing if the color of the medium turn yellowish

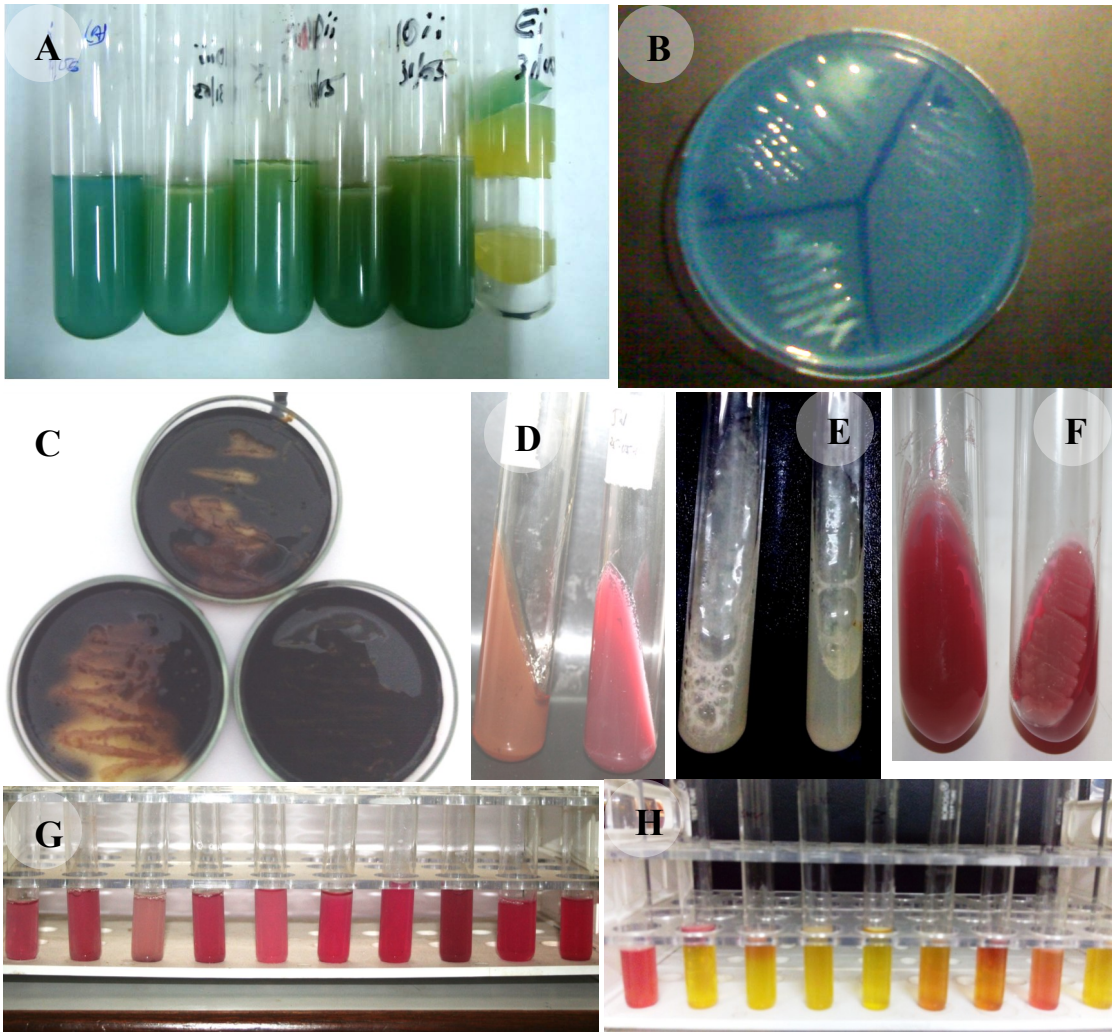

Fig. 4.6 Result of various biochemical tests (see text for details) (A) Acid alkaline production test (B) Methylene blue test (C) Starch hydrolysis test (D)Triple sugar iron agar test (E) Catalase test (F) Urease test (G) Nitrate reduction test (H) Carbohydrate utilization test

(Somasegaran and Hoben, 1994).

In this present study, when the pH value of the broth was measured after the color change, a decrease in pH of the culture broth was recorded (Table 4.2). The pH of BIJ isolate was found to be 5.8 and that of RSm-3 to be 6.87. Hence, all the *Rhizobium* strains were found to be acid producers. The production of acid has been found to be a characteristic of *Rhizobium* (De Vries *et al*. 1980; Bao Ling *et al*. 2007; Singh *et al*. 2008).

4.6.4.Glucose peptone agar (GPA) test

There is a common belief that proper growth in glucose containing medium is a confirmatory test for *Rhizobium* (Singh *et al*. 2008). However, it has been found that majority of the strains like DJG, KPG-5N, MIR-6, SKM-N, SKM-W, SKM-E, NBU-8, RSv-1, RMa-13 and RGJ could not grow in the GPA medium (refer table 4.2). Deshwal and Chaubey, (2014) have also reported the failure of all the strains of *Rhizobium leguminosarum* isolated from the root nodules of *Pisum sativum* to grow in the medium. Similarly, no growth was found in the culture of *Rhizobium* isolated from root nodules of Mung bean (*Vigna radiata* L.) (Bhatt *et al*. 2013). Hence, the utilization of glucose as a carbon source may not be considered as a confirmatory test for *Rhizobium*.

4. 6. 5. Methylene blue test

Methylene blue is used as an agent against the growth of the microorganisms and workers like Singh *et al*. (2008) and Wei *et al*. (2003) found the bacteria to be sensitive at 0.1% of dye. However, in contrast to their findings, it was found that all the rhizobial strains under study grew on medium containing 0.1% dye (Fig. 4.6 B). The possible explanation may be due to the vast genetic diversity among the strains

4.6.6. Starch hydrolysis test

Except RSm-3, RMa-13 and RSv-1 strains, no halo formation around the growing area of the strains was observed after the addition of iodine on any of the SAM plates. The dark blue coloration of the medium indicated the non-utilization of starch (Fig. 4.6 C). Deshwal and Chaubey (2014) also noted a negative starch hydrolysis for all the strains of *R. leguminosarum* isolated from the root nodules of *Pisum sativum*. Similarly many other workers also noted such negative starch hydrolysis test elsewhere while working with different rhizobial strains

isolated from root nodules of different legumes (Deka and Azad, 2006; Gachande and Khansole, 2011).

4.6.7. Gelatin hydrolysis

No liquefaction of the gelatin was recorded after the tubes were brought out from refrigerator kept at 4ºC. This indicated the absence of gelatinase enzyme, a proteolytic exoenzyme produced by some microorganisms that has the potential to hydrolyze or liquefy the gelatin (Aneja, 1996). Negative gelatinase activity of *Rhizobium* has been reported by several workers supporting this present finding (Hunter *et al*. 2007; Singh *et al*. 2008; Gachande and Khansole, 2011; Bhatt *et al*. 2013; Deshwal and Chaubey, 2014).

4.6.8. Triple sugar iron (TSI) agar test

The TSI test was performed to check the ability of microorganism to ferment the sugars like lactose, glucose and sucrose by changing the color of slant and butt (Hajna, 1945). According to Singh *et al.* (2008) three possible observations could occur on the basis of capability of organisms to use carbohydrate:

(i) acid (yellow) slant and acid (yellow) butt indicated the lactose and sucrose fermentation

(ii) alkaline (red) slant and acid (yellow) butt indicated the glucose fermentation, whereas (iii) the alkaline (red) slant and alkaline (red) butt indicated no carbohydrate fermentation.

In this study, most of the isolates could not grow in TSI agar medium. However, alkaline (red) slant and acid (yellow) butt was observed in few strains like SKM-N, SKM-W, SKM-E, NBU-8, RSv-1, BIJ, RMa-13 and RGJ strains indicating glucose fermentation (Fig 4.6D). Only one isolate MIR-6 showed red slant and red butt indicating no carbohydrate fermentation.

4.6.9. Catalase test

No effervescence in the culture tubes were observed on the addition of 3% H_2O_2. This was due to absence of the enzyme catalase, which breaks down hydrogen peroxide (H_2O_2) to water and oxygen protecting the cell from oxidative damage by reactive oxygen species (ROS) helping in survival of the cell (Chelikani *et al*. 2004). Deora and Singhal (2010) also found the *Rhizobium* strains to be unable to produce catalase during their work. However, similar to Deshwal and Chaubey (2014) and Datta *et al.* (2015)

strains like RSm-3, RMa-13 and RSv-1 strains showed a positive catalase test (Fig. 4.6E).

4.6.10. Urease test

A change in color of the media from yellow to pink was observed (Fig. 4.6 F). The color change may be due to the degradation of urea to ammonia by urease enzyme secreted by the bacteria in the medium. The formation of ammonia alkalinizes the medium, and the pH shift is detected by the color change of phenol red from light orange at pH 6.8 to magenta (pink) at pH 8.1. Thus, all the isolates showed a positive test for urease. Similar, observation was reported by many workers elsewhere working with different legumes (Bedi and Naglot, 2011; Datta *et al.* 2015).

4.6.11. Cellulase production test

Cellulase is an enzyme which degrades the cellulose in the medium. The microbial utilization of cellulose is detected by using HDTMA. This reagent precipitates the intact Carboxymethyl cellulose (CMC) in the medium creating a clear zone around the colony indicating degradation of CMC.

In this study, majority of the strains like K-1, RSm-3, KPG-5N, MIR-6, SKM-N, SKM-W, SKM-E, NBU-8, RSv-1, BLG, RMa-13 and RGJ could not grow in the medium barring a few like DJG, SAM-12, CBR, BIJ and JPG. However, no clear zone was found around the colonies upon addition of 1% solution of HDTMA in the growth medium, indicating that no cellulase production took place. According to Dhingra and Priya (2013) negative cellulase production is a common phenomenon shown by the *Rhizobium* strains.

4.6.12. Nitrate Reduction test

A red color change of the medium was observed on the addition of the reagents like sulphanilic acid and α-naphthylamine. The production of the enzyme nitrate reductase by the bacteria in the medium led to the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) which reacts with the reagents to form a red color (Cappuccino and Sherman, 2008). In this study, experiments majority of the isolates were found to be positive for nitrate reduction except for isolates like MIR-6 and JPG (Fig. 4.6F). The result was similar to those of workers like Graham and Parker (1964); Salve and Gangwanae (1992); and Kumari *et al.* (2010).

4.6.13. Carbohydrate utilization test

In one experiment, when phenol red was added to the bacterial incubated nutrient broth with 0.1% sugar, a change to yellowish colour was observed indicating the fermentation of sugars (Fig. 4.6G).

In the other study, all *Rhizobium* strains were subjected to grow in the minimal MOPS-salt (MS) medium (refer materials and method section 3.7.13) amended with five sugars individually namely, fructose, dextrose, sucrose, lactose, mannitol and five sodium organic salts like sodium acetate, sodium succinate, sodium citrate, sodium propionate and sodium pyruvate.

The utilization of a wide range of carbohydrates and salts of organic acids as carbon sources has been reported by various workers like Jordan, (1984) Rodriguez-Navarro *et al.* (2000), Zerhari *et al*. (2000), Kucuk and Kivanc, (2008), Bedi and Naglot, (2011) etc.

Considering the presence or absence of growth of the *Rhizobium* strains in various conditions, grouping of the strains were done using NTSYS pc software. As expected, the strains were found to club together according to their tendency of utilizing certain sugars and organic salts over others.

The dendrogram revealed the formation of three clusters at a level of 0.65 (Fig. 4.7). In cluster I, the strains flocked together in one common group

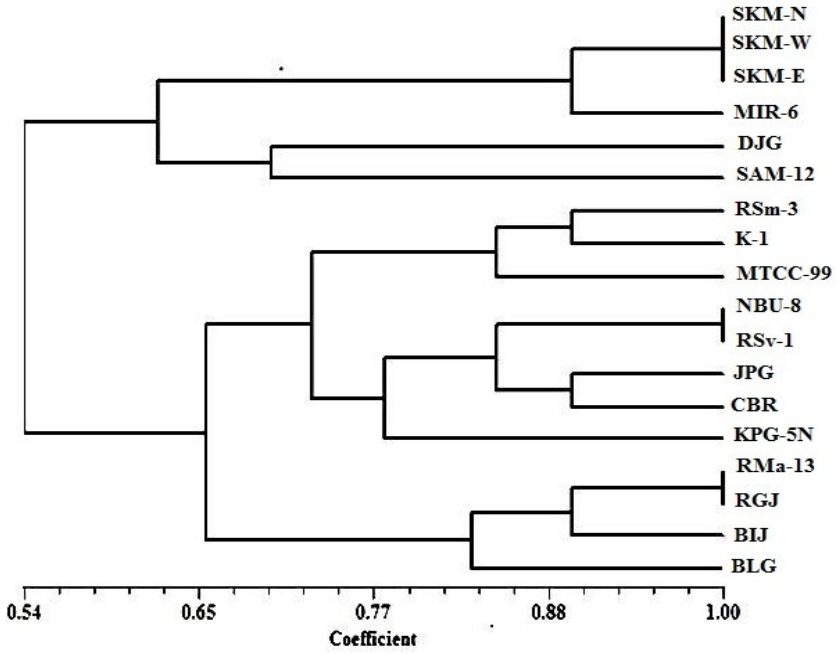

Fig. 4.7 Dendrogram of *Rhizobium* strains generated by NTSys program based on the carbohydrate utilization test

on the basis of their affinity towards sugars like dextrose and sodium acetate. The strains comprised from Sikkim (SKM-N, SKM-W, SKM-E), Mirik (MIR-6), Darjeeling (DJG) and Samsing (SAM-12). However, the first four strains also utilized fructose while DJG and SAM-12 utilized mannitol, sodium succinate, sodium pyruvate and sodium propionate as well.

Cluster II consisted of strains which were able to utilize a broad range of sugars like fructose, dextrose, sucrose, sodium acetate and sodium propionate in common, thus flocking together to form a larger group. The strains like RSm-3, K-1, MTCC-99, NBU-8, RSv-1, CBR and KPG-5N belonged to the category. However, the JPG strain was an exception as it could only utilize fructose, dextrose and sucrose as a carbon source.

The third cluster consisted of mainly the strains utilizing sodium pyruvate as the carbon source. Although fructose, dextrose, sucrose, sodium acetate and sodium propionate were also used by the strains like RMa-13, RGJ, BIJ and BLG.

Thus, although the grouping of the strains could be achieved based on their utility of the sugars and organic salts, the strains did not show uniformity in the utilization pattern. These findings were in corroboration with that of Deka and Azad (2006).

To summarize the details, the most common carbon sources utilized by all *Rhizobium* strains were found to be dextrose and fructose. Among the organic salts, sodium acetate was used by all the strains followed by sodium propionate. Athough, the utilization of succinate, citrate, fumarate, malate and pyruvate as a carbon source has been found to be an important characteristic in case of different strains of *R. leguminosarum* and *R. meliloti* (Jordan 1984), sodium citrate was found to be the least utilized organic salt in comparison to others.

4.7. Physiological characterization

4.7.1. Sodium chloride tolerance test

The strains were subjected to 1-4% of sodium chloride to check their tolerance level. The OD values of the cultures were recorded at 540nm after 48 hr. of growth The KyPlot of the OD values of the growing strains revealed the decrease in the level of growth with the increase in the concentration of sodium chloride. A statistically significant difference was obtained from the ‘t-test’ (Fig. 4.8 Ai-Aii).

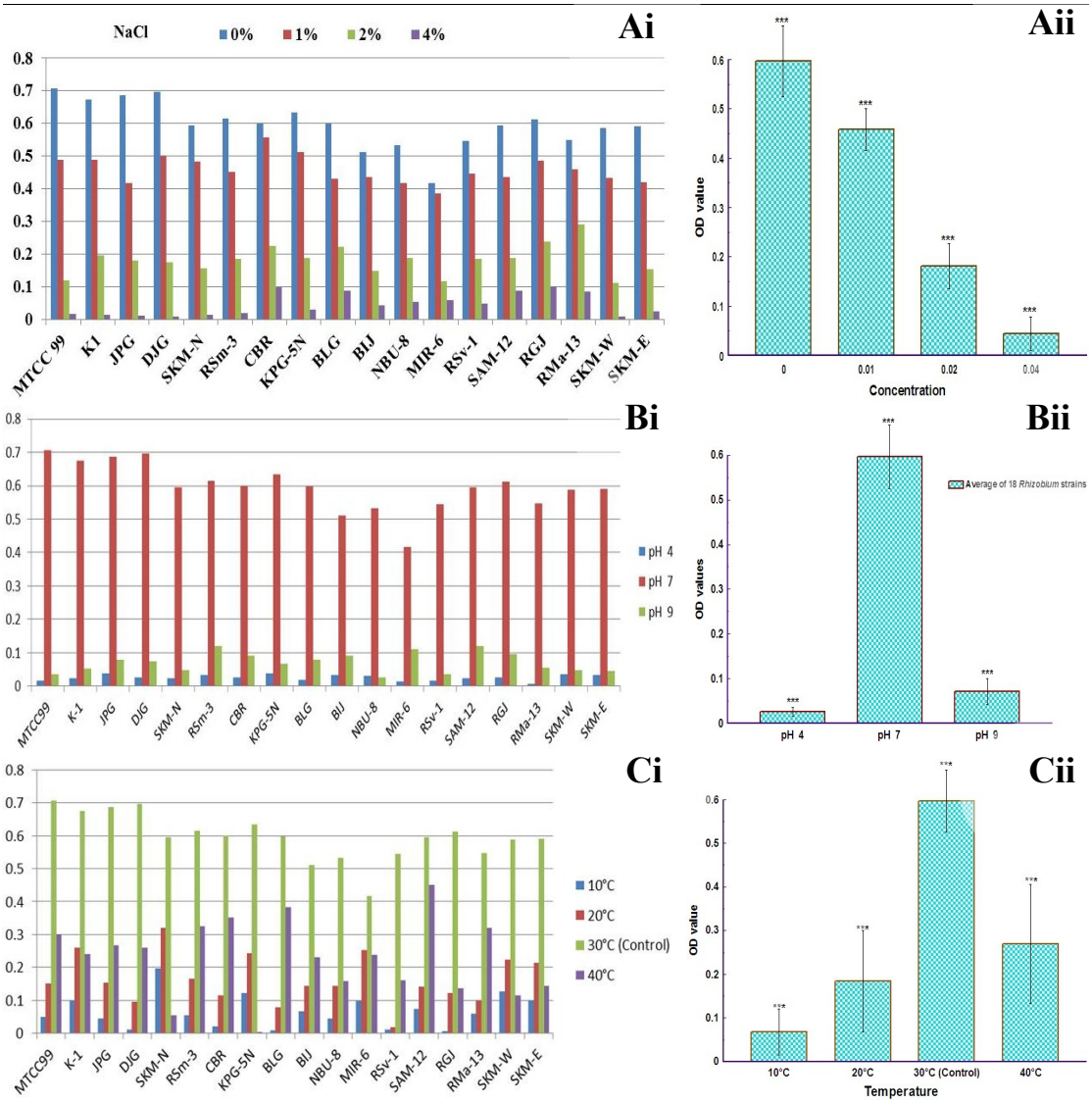

Fig.4.8 Ai: Growth of *Rhizobium* strains grown in different NaCl concentration; Aii: KyPlot representing the statistical data of the *Rhizobium* growth in different NaCl concentration; Bi: Growth of *Rhizobium* strains grown in different pH; Bii: KyPlot representing the statistical data of the *Rhizobium* growth in different pH concentration; Ci: Growth of *Rhizobium* strains grown in different temperature; Cii: KyPlot representing the statistical data of the *Rhizobium* growth in different temperature.

All the strains grew in 1% NaCl but variations in growth occurred as concentration of the salt increased. The growth of the studied strains like CBR, BLG, RGJ and RMa-13 was found to be fairly high compared to other strains. However, growth of the strains decreased at 4% NaCl, although the strains CBR, BLG, RGJ and RMa-13 showed some amount of growth even at such high concentration. Datta *et al* (2015) found all the strains of *Rhizobium* like *R. phaseoli, R. leguminosarum* and *R. trifolii* to

tolerate 0.5-4 % of the salt level.

4.7.2. pH tolerance test

Among the three different pH range 4, 7 and 9, the growth of the studied strains were found to be the maximum in pH 7 (Fig.4.8Bi-Bii). The OD values of the cultures ranged from 0.417-0.707. In comparison to the lower pH 4, the growth of the strains was found to be comparatively higher at pH 9. The OD values of the cultured strains like CBR, BIJ and RGJ were highest at pH 9 compared to other strains. However, the growth of all the strains was found to be very low at pH 4.

The 't test' revealed the growth of the strains at pH 4 and pH 9 to be statistically significant to the growth at pH 7 at *p* value of less than or equal to 0.001.

4.7.3. Temperature tolerance test

The *Rhizobium* strains were subjected to various temperature gradients 10ºC, 20ºC, 30ºC and 40ºC in order to check their temperature tolerance. The KyPlot showed maximum growth of all the strains at 30ºC while the growth decreased with the increase and decrease in temperature (Fig. 4.8 Ci-Cii). However, strains like KPG-5N, SKM-E, MIR-6 and SKM-W showed a good amount of growth at 20ºC and even at 10ºC. Similarly, some of the strains like JPG, CBR, BLG and NBU-8 grew well at a higher temperature of 40ºC as well. The 't-test' showed a statistically significant result at *p* value of less than or equal to 0.001 in growth level of the strains at the varying temperature. There have been reports of growth of *Rhizobium meliloti* at a higher temperature like 42.5ºC by workers like Jordan (1984) and Datta *et al*. (2015).

4.7.4. Heavy metal resistance test

Three types of heavy metals $CuSO_4$, $Pb(NO_3)_2$ and $CoCl_2$ were used to study the effect of these heavy metals on the growth of the *Rhizobium* strains. For this study, six different concentrations 0.01mM, 0.1 mM, 0.5 mM, 1 mM, 2 mM and 4 mM of these heavy metals were taken into account.

It was observed that with the increase in the concentration of heavy metals, a sudden fall in the growth of the strains was observed. The KyPlot indicated that the heavy metal $Pb(NO_3)_2$ was the least potent inhibitor to the *Rhizobium* growth, as some of the strains like K-1, DJG, RSm-3 and NBU-8 happened to grow at 3mM concentration as well (Fig. 4.9A-C). Similar result was obtained by El-Deeb and Al-Sheri

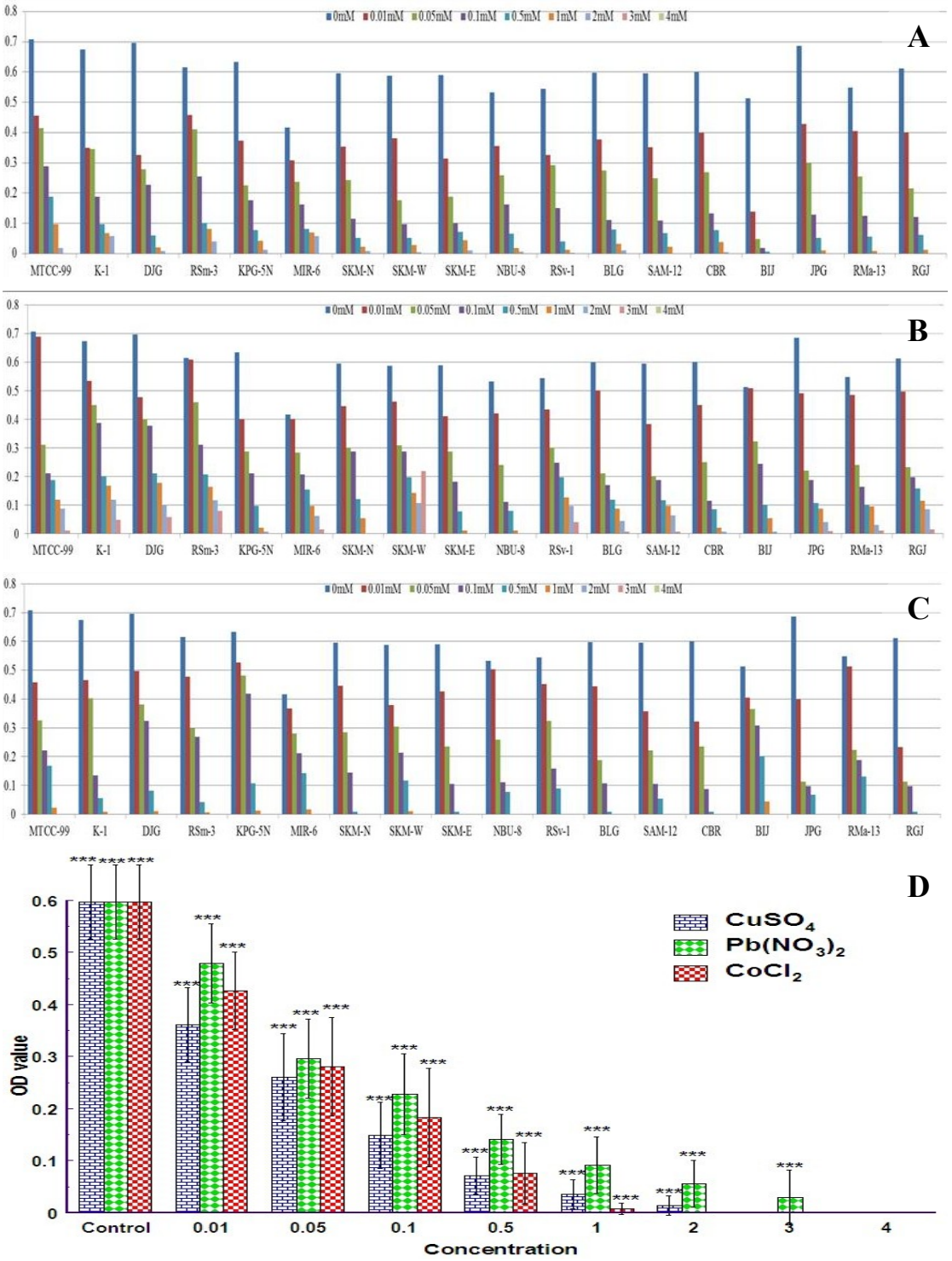

Fig.4.9.Growth of *Rhizobium* strains in different concentration of heavy metals (A)CuSO4, (B)Pb $(NO_3)_2$, (C)$CoCl_2$. X-axis represents the strains and Y-axis represents the OD values. (D) KyPlot representing the statistical data of the *Rhizobium* growth in different heavy metal concentration.

(2005). However, $CoCl_2$ emerged as the most toxic of the heavy metal as most of the strains could tolerate a concentration of 0.5mM. Only one or two strains like MTCC-99 and BIJ could grow at a concentration of 1mM.

Similarly, the growth of the strains decreased with the increase in the concentration of the heavy metal $CuSO_4$ (Fig. 4.9A). However, some strains like K-1, RSm-3 and MIR-6 showed a fair amount of growth in *2*mM while, majority of the strains were unable to grow. However, not a single strain could grow at 4mM concentration of any of the heavy metals.

In this experiment cobalt (Co) was found to be the most toxic of the heavy metal and lead (Pb), the least potent inhibitor to the *Rhizobium* growth (El-Deeb and Al-Sheri, 2005). The order of toxicity of the heavy metals was found to be Co >Cu>Pb. A statistically significant result based on the growth of strains at different concentration of heavy metals were obtained by 't-test' at a *p* value of less than or equal to 0.001 (Fig. 4.9D).

There have been several reports by earlier workers depicting the fast growing rhizobia to be sensitive to heavy metals than the slow growers (Maatallah *et al.* 2002; El-Akhal *et al*. 2009). However, in this study French bean rhizobial isolates which are all fast growers, showed a wide range of behavior with regard to the resistance to heavy metals. The results indicated that the tolerance of strains to heavy metals is not depicted by their growth rate, but it could be due to the genetic variation among the bacterial species isolated from different locations.

4.7.5. Intrinsic antibiotic resistance (IAR) test

In this study, the agar disc diffusion test of Bauer *et al*. (1966) was followed for the IAR test, as it is regarded to be the most convenient and widely used method for routine antimicrobial susceptibility testing. Each of the strains showed a wide variation in their result in response to different concentration of 40 antibiotics (Fig. 4.10). However, it was not possible to tally the antibiotic profile for 6 different antibiotics as the standards were not available for rest of

Fig.4.10. IAR test showing the zone of inhibition by the *Rhizobium* strain RSm-3.

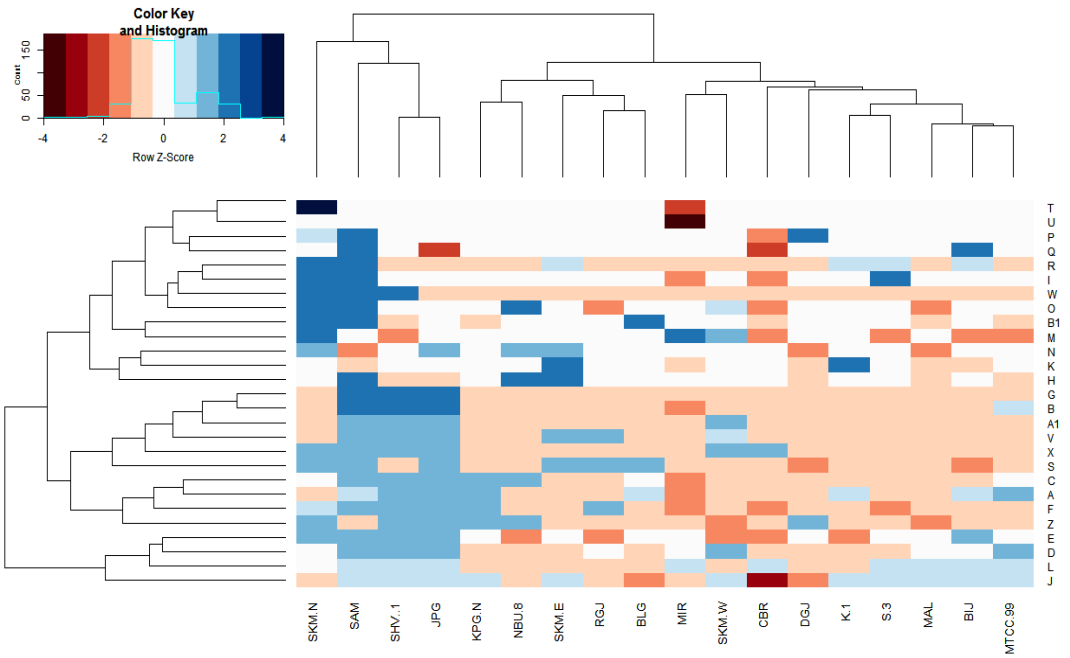

Fig.4.11. Heatmap of the antibiotic resistance property of studied *Rhizobium* strains . The color ranged from dark brown (very resistant) to dark blue (sensitive).

the antibiotics, please refer table 3.2 (Materials and method section).

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. The color ranged from dark brown (very resistant) to dark blue (sensitive).

From the heatmap, it was found that the studied *Rhizobium* strains were divided into three clusters depending upon the sensitivity against their antibiotics (Fig 4.11).

In the first cluster (cluster A) four strains were there viz., SKM-N, SAM-12, RSv-1 and JPG. These strains have been found to be mostly susceptible against various antibiotics. On the other hand, Cluster C consisted of 9 strains including the reference strain MTCC-99. The strains were MIR-6, SKM-W, CBR, DJG, K-1, RSm-3, RMa-13 and BIJ. These strains were more or less resistant against the bunch of antibiotics used. It was found that some of them were highly resistant against certain antibiotics. For instance, the *Rhizobium* strain MIR-6 was highly resistant against Norfloxacin (Nx) and Ciprofloxacin (Cf) while the strains CBR was resistant against Cefadroxil (Cq).

The cluster B was somewhat intermediary between cluster A and

cluster C having mixtures of relatively low resistant strains. There were five strains in this category and those were KPG-5N, NBU-8, SKM-E, RGJ and BLG.

Attempts have been made to correlate the antibiotics based on their classifications (i.e. aminoglycosides, amphenicol, carbapenem, cephalosporin, glycopeptide, hydantoin, macrolide, penicillin, polypeptide and quinolones) with resistance patterns of the strains. However, no significant correlation was detected. Also, it has been found that the clustering of *Rhizobium* strains based on IAR profiles did not reflect their geographical origin. Similar conclusion was drawn by Alexandre *et al.* (2006) while working on chickpea rhizobia.

4.8. Plant growth promoting traits

Plant growth promoting rhizobacteria (PGPR) promotes plant growth either directly (nitrogen fixation, phosphate solubilization, iron chelation and phytohormone production) or indirectly (suppression of plant pathogenic organisms, induction of resistance in host plants against plant pathogens and abiotic stresses) (Gopalakrishnan *et al*. 2015).

In this study, several *in vitro* tests were performed to check the potential of *Rhizobium* as PGPR.

4.8.1. in –vitro PGPR study

4.8.1.1. Phosphate solubilization activity

The appearance of transparent halo zone around the colony grown in Pikovskaya's agar medium indicated the phosphate solubilizing activity of the strain (Pikovskaya, 1948).

Such clear halo zone was observed in studied strains like MTCC-99, DJG, RSm-3, KPG 5N, SKM- N, RSv-1, SAM-12, CBR, BIJ, RMa-13 and RGJ (Fig.4.12 A). The halo-zone diameter measured from the centre of the colony varied from 1.6 cm in KPG-5N to 2.36 cm in RGJ. The Phosphate solubilizing efficiency (PSE) of DJG strain was found to be the highest (66.67) and the lowest was found in RSm-3 i.e. 35.89. However, strains like K-1, MIR-6, SKM-W, SKM-E, NBU-8, BLG and JPG could not solubilize phosphate. This present result was in corroboration to Kumar *et al*. (2012) who screened a total of thirty bacterial isolates for phosphate solubilization on modified PVK agar, of which only twelve isolates showed the development of sharp phosphate

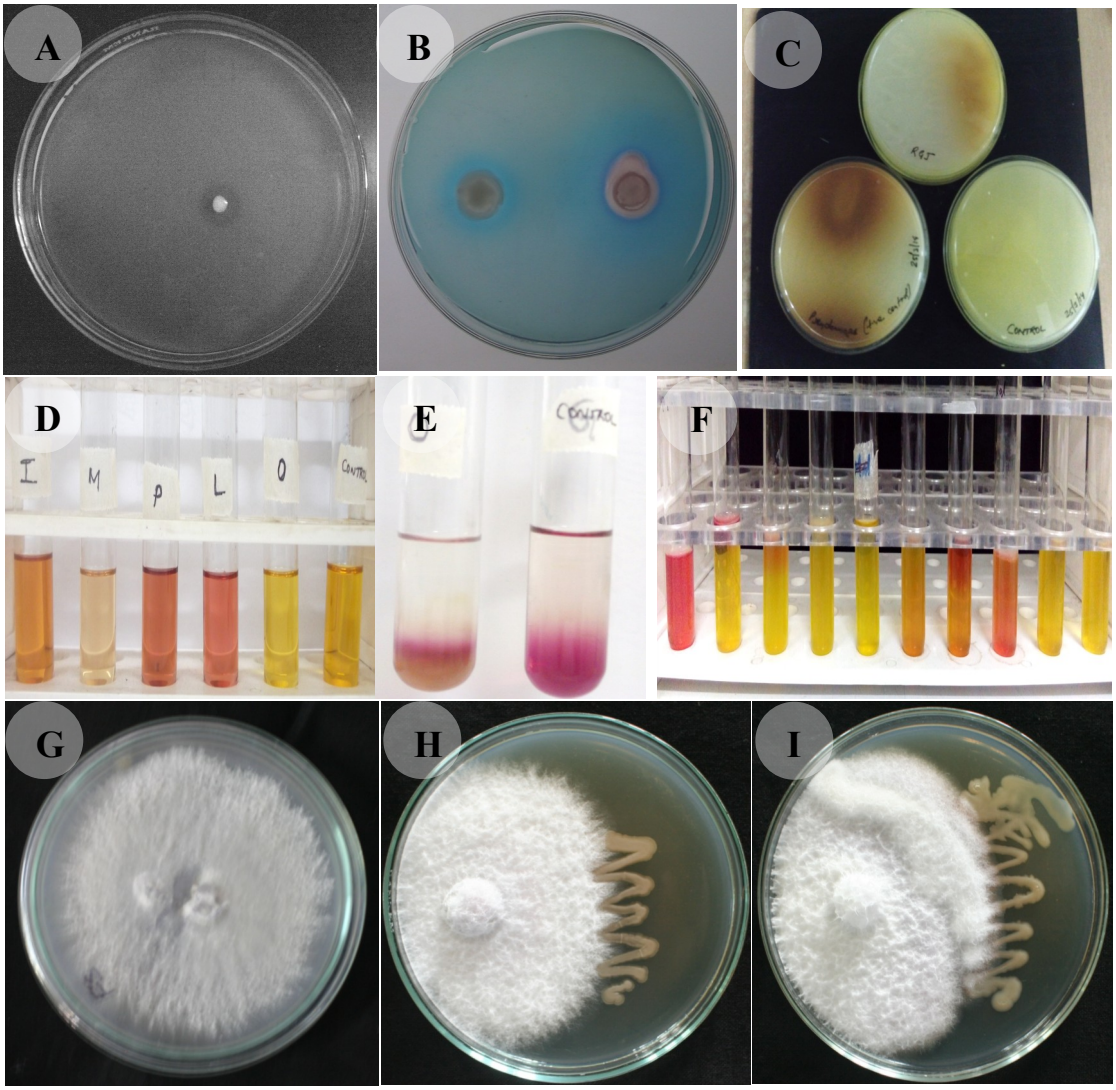

Fig 4.12 (A) Halo zone formation shown by the *Rhizobium* strains indicating phosphate solubilization activity, (B) *Rhizobium* strains showing siderophore producing activity, (C) *Rhizobium* strains showing HCN producing activity, (D,E) *Rhizobium* strains showing IAA producing activity, (F) *Rhizobium* strains showing ammonia producing activity, (G) Plate showing pure culture of fungus *Fusarium solani,* (H,I) *Rhizobium* strain RSm-3 and *Rhizobium* strain NBU-8 showing antagonistic activity against the fungus *Fusarium solani* respectively.

solublization zones.

4.8.1.2. Siderophore production activity

Siderophore production test using CAS agar plate has been found to be useful for rapid screening of potential beneficial bacterial isolates. The bacteria producing siderophores, enhances the plant growth by increasing the availability of iron near the roots (Alexander and Zuberer, 1991; Tan *et al.* 2014).

An orange halo colored zone was observed in the CAS–agar medium inoculated with the strains depicting siderophore production. Out of the seventeen isolates including the reference strain MTCC-99 screened for

Table 4.3. Results of *in vitro* PGPR properties of the *Rhizobium* strains

Isolates	IPT	PST				SPT	HPT	Aa
	µg/ml	+/-	Cd (mm)	HZD (mm)	PSE%			
MTCC99	55.7±1.5	+	12.7±0.15	19.3±0.24	52.63	-	+	+
K-1	20.0±1.7	-	12.7±0.09	0	0	-	-	-
DJG	75.7±0.9	+	11.0±0.12	18.3±0.07	66.67	+	+	+
RSm-3	19.3±1.3	+	13.0±0.15	17.7±0.22	35.90	-	+	+
KPG-5N	7.7±0.9	+	11.7±0.09	16.0±0.10	37.14	+	+	+
MIR-6	26.3±1.2	-	13.3±0.12	0	0	-	-	-
SKM-N	25.0±1.7	+	13.7±0.09	20.7±0.09	51.22	+	+	+
SKM-W	20.0±1.5	-	12.7±0.09	0	0	-	-	-
SKM-E	18.3±1.5	-	15.7±0.09	0	0	-	-	-
NBU-8	8.3±1.2	-	13.7±0.09	0	0	-	-	-
RSv-1	32.3±1.5	+	13.0±0.15	2.0±0.21	53.85	+	+	+
BAL	19.3±0.7	-	14.3±0.15	21.3±0.12	48.84	-	-	-
SAM-12	32.3±0.9	+	12.0±0.21	17. 7±0.09	47.22	+	+	+
CBR	41.7±1.2	+	16.0±0.06	22.0±0.15	37.5	-	+	+
BIJ	45.7±0.9	+	15.3±0.09	21.7±0.20	41.30		+	+
JAL	7.7±0.9	-	12.0±0.10	0	0	-	-	-
RMa-13	43.0±1.0	+	12.0±0.12	17.0±0.12	41.67	+	+	+
RGJ	7.7±1.5	+	16.0±0.12	23.7±0.15	47.92	+	+	+

IPT=Indole acetic acid production test; +/-= Present/absent; Cd= Colony diameter;HZD= Halo zone diameter; PSE= Phosphate solubilization efficiency; SPT= Siderophore production test; HPT= Hydrogen cyanide production test; Aa= Antagonistic activity

the siderophore production test, only six isolates DJG, KPG-5N, NBU-8, RSv-1, SAM-12 and RMa-13 were found to be siderophore producers (Fig. 4.12B). Thus, only 33.33% of the total isolates were found to be siderophore producers.

Similarly, Derylo *et al.* (1994) found that out of 84 isolates collected from different legumes only nine isolates gave positive reactions with CAS.

4.8.1.3. HCN production activity

After the incubation of inoculated plates for 3-4 days, the yellow colored picric acid soaked filter paper changed brownish in coloration. This indicated the production of hydrogen cyanide by the bacteria.

However, not all the strains were found to be positive in producing HCN. The isolates found to be positive for

hydrocyanic acid production test were MTCC-99, DJG, RSm-3, KPG-5N, SKM-N, NBU-8, RSv-1, SAM-12. , CBR, BIJ, RMa-13 and RGJ (Fig. 4.12C). According to Antoun *et al*. (1998) such variation is common in rhizobia .

4.8.1.4. IAA production

The appearance of pink color produced after exposure of broth to Salkowski reagent distinguished the *Rhizobium* strains as IAA producers from other indole producing organisms. Among the 18 strains including MTCC-99, the indication of being IAA producer by its color change were DJG, RSm-3, KPG-5N, SKM-N, RSv-1, SAM-12, BIJ, RMa-13 and RGJ (Fig 4.12D-E). The absorbance of the resultant pink color of the IAA producers was read at 535 nm in UV/visible spectrophotometer and the IAA production calculated from the regression equation of standard curve. The range of IAA production was recorded to be 7.67 ±1.45 to 75.67 ±0.88 µg/ml. The DJG strain was found to be the highest IAA producer (75.67±0. 88 µg/ml) and RGJ, the lowest producer (7.67±1.45 µg/ml).

4.8.1.5. Ammonia production test

Another important trait of PGPR is the production of ammonia that indirectly influences the plant growth. The incubation of the bacteria in peptone water broth for 48 hr was followed by the addition of few drops of Nessler's reagent. A change from transparent to yellow coloration is indicative of ammonia production. In this study all the *Rhizobium* strains were found to be positive for ammonia production test (Fig.4.12F). Similar result was observed by (Singh *et al*. 2013).

4.8.1.6. In vitro antagonistic activity

It has been reported that different species of *Rhizobium* can significantly reduce wilt and root rot disease of common bean and chickpea caused by *Fusarium solani* (Chao, 1990; Dar *et al.* 1997; Ozkoc and Deliveli, 2001; Kucuk, 2013) acting as a biocontrol agent. The competition for nutrients, secretion of antibiotics and cell wall degrading enzymes as well as secretion of certain toxic, secondary metabolites, antimicrobial and bacteriocin like substances may be the reason behind the antagonism (Nirmala and Gaur 2000; Arora *et al.* 2001; Hafeez *et al*. 2005; Kucuk, 2013).

The antagonistic activity of *Rhizobium* against the fungal pathogen *F. solani* was recorded after 7 days of

incubation. The result revealed that majority of the isolates like MTCC-99, DJG, RSm-3, KPG-5N, SKM-N, SAM -12, CBR, BIJ, RMa-13 and RGJ showed antagonistic activity against the fungal pathogen (Fig. 4.12 G, H & I) while the remaining isolates K-1, BLG, NBU-8, MIR-6, RSv-1, SKM-W and SKM-E failed to inhibit the growth of the pathogen.

The details of the results of the *in vitro* PGPR study of the studied *Rhizobium* strains has been presented in Table 4.3.

4.8.2. in vivo greenhouse study

On the basis of the *in vitro* PGPR activities eleven potential strains such as DJG, RSm-3, KPG-5N, SKM-N, RSv-1, SAM-12, CBR, BIJ, RMa-13, RGJ and MTCC 99 were selected for the *in vivo* study. In this study, the greenhouse experiment was conducted to evaluate the effect of *Rhizobium* inoculation on different growth parameters of French bean (Fig. 4.13 A -B).

The bacterization of French bean seeds were done with the selected rhizobial strains using charcoal as a carrier material which has been found to be an effective carrier medium as that of peats (Sparrow and Ham, 1983). The sterility of the soil was maintained by regular watering with sterile water. After 45 days of sowing, the data generated were recorded. The statistical analysis was done by comparing the mean of the data using Duncan's multiple range test (DMRT) at p = 0.05 with Statistica software version 5.0 (Statsoft 1995).

A statistically significant difference was found within different parameters like plant height, shoot fresh weight, shoot dry weight, root fresh weight and root dry weight obtained by the bacterial seed treatment in comparison to the control treatment without inoculation (table 4.4). However, not much statistically significant differences were observed within the leaf length, leaf breadth and root length. It was found that the growth parameters of the plant viz. pant height, shoot fresh weight, shoot dry weight, root fresh weight and root dry weight, treated with DJG strain was found to be the maximum compared to the other strains as well as the uninoculated control.

The shoot dry weight yield varied from 0.623 gm^{-1} plant to 1.28 gm^{-1} per plant which was significantly higher than that of T0 (0.49 gm^{-1}plant). The highest shoot dry weight, as the main indicator of nitrogen fixing ability and

Fig 4.13 (A) Greenhouse pot experiment showing forty-five days old *P. vulgaris* inoculated with different *Rhizobium* strains. (B) *P. vulgaris* during the flowering stage.

Table 4.4. The effect of *Rhizobium* inoculation on different growth parameters of French bean (*Phaseolus vulgaris* L.) in green house condition.

TM	PH (cm)	LL (cm)	LB (cm)	SFW	SDW	RL (cm)	RFW	RDW	NN
T0	137.67±1.45h	6.17±0.60d	5.50±0.29d	1.85± 0.04i	0.49± 0.01i	19.67±1.2f	1.13±0.65f	0.34±0.012d	0.00i
MTCC 99	173.67±1.20d	9.23±0.15b	8.00±0.58a	2.84± 0.05f	0.82± 0.02e	31.00±1.53bc	1.22±0.71d	0.55±0.02b	28.33±2.03g
DJG	188.33±0.88a	10.87 ±0.38a	7.87±0.08a	4.18± 0.03a	1.28± 0.02a	31.67±2.61abc	2.35±0.56a	0.67±0.01a	94.67±4.81a
RSm-3	162.67±1.20e	7.67±0.88c	6.83±0.44abc	2.61± 0.02g	0.72± 0.02g	25.00±0.58e	1.33±1.36c	0.40±0.012c	43.00±2.08d
KPG-5N	182.33±1.45b	8.33±0.44bc	7.33±0.44ab	3.87± 0.04b	1±0.02b	31.33±2.19bc	1.05±0.61g	0.23±0.01f	58.00±2.08c
SKM-N	161.33±0.88e	8.17±0.73bc	7.17±0.44ab	3.51± 0.05c	0.91± 0.02c	32.67±2.61ab	1.17±0.68e	0.31±0.01de	73.33±1.77b
RSv-1	155.33±1.45g	7.50±0.29cd	6.33±0.17bcd	2.47± 0.03h	0.71± 0.02g	27.33±1.86cd	1.53±0.88b	0.41±0.02c	26.00±3.06g
SAM-12	176.67±1.33c	9.13±0.32b	7.15±0.32ab	3.79±0.04b	1±0.01b	25.00±1.73e	0.88±0.51i	0.30±0.01e	19.33 ±0.88h
CBR	163.33±1.77e	8.08±0.61bc	6.33±0.60bcd	2.61±0.04g	0.76± 0.01f	30.00±1.53bc	1.00±0.58h	0.23±0.01f	35.00±1.73ef
BIJ	153.33±1.33g	7.23±0.43cd	5.67 ±0.73cd	2.44± 0.03h	0.62± 0.01h	32.33±0.88ab	0.85±0.49i	0.14±0.01h	36.00±1.53de
RMa-13	158.00±1.16f	7.50±0.29cd	6.50±0.29bcd	3.36± 0.07d	0.877± 0.01c	26.33±0.88de	1.09f±0.63g	0.19±0.01g	34.33±2.19f
RGJ	162.67±1.2e	7.17±0.44cd	7.42±0.46ab	3.32± 0.02e	0.86± 0.02cd	36.67±1.77a	1.32±0.76c	0.34±0.01d	56.33±2.97c
CD	**3.929**	**1.428**	**1.25**	**0.119**	**0.04**	**4.55**	**0.048**	**0.038**	**7.101**
SE(d)	**1.882**	**0.684**	**0.599**	**0.057**	**0.019**	**2.18**	**0.023**	**0.018**	**3.402**
SE(m)	**1.331**	**0.484**	**0.424**	**0.04**	**0.014**	**1.542**	**0.016**	**0.013**	**2.406**
CV	**1.401**	**10.359**	**10.724**	**2.272**	**2.82**	**9.18**	**2.298**	**6.503**	**9.914**

TM= Treatment; PH= Plant Height; LL= Leaf length; LB= Leaf Breadth; SFW= Shoot fresh weight^{-1}; SDW= Shoot dry weight^{-1}; RL= Root length; RFW= Root fresh weight^{-1}; RDW= Root dry weight^{-1}; NN= Nodules number^{-1}; T0- Uninoculated control; CD= Critical Difference; SE (d)= Standard error; SE (m)= Standard error of mean; CV= Critical variance

Table 4.5. Correlation between different growth parameters of French bean treated with 11 Rhizobium strains

	PH	LL	LB	SFW	SDW	RL	RFW	RDW	NN	OC	N_2	KO	PO	AN_2
PH	1.000													
LL	.853**	1.000												
LB	.768**	.686*	1.000											
SFW	.798**	.723**	.746**	1.000										
SDW	.800**	.730**	.730**	.996**	1.000									
RL	.184	.109	.477	.318	.311	1.000								
RFW	.116	.119	.413	.102	.070	.091	1.000							
RDW	.262	.320	.473	.069	.054	-.046	.877**	1.000						
NN	.424	.315	.522	.560	.525	.736**	.280	.049	1.000					
OC	.489	.605*	.336	.293	.299	-.084	-.077	.111	.186	1.000				
N_2	.538	.664*	.414	.295	.307	-.037	.007	.269	.179	.960**	1.000			
KO	.624*	.481	.205	.447	.466	.373	-.190	-.131	.409	.147	.158	1.000		
PO	-.025	-.032	.259	.011	-.021	-.130	.290	.399	-.176	-.139	-.105	-.356	1.000	
AN_2	.786**	.730**	.595*	.675*	.654*	.254	.193	.209	.722**	.645*	.646*	.512	-.040	1.000

PH= Plant height; LL= Leaf length; LB=- Leaf breadth; SFW= Shoot fresh weight; SDW= Shoot dry weight; RL= Root length; RFW= Root fresh weight; RDW= Root dry weight; NN= Nodule number; OC= Organic carbon content; N_2= Soil nitrogen content; KO= Soil potassium content; PO= Soil phosphorus content; AN_2= Aerial shoot nitrogen content

growth promotion, was detected in plant co-inoculated with DJG strain. Several reports showed that shoot dry mass is a good indicator of relative strain effectiveness, and there is a good correlation between shoot dry matter production and nitrogen fixation capacity of legumes (Somasegaran and Hoben, 1994; Peoples *et al.* 2002; Belay,2006).

Table 4.6. Analysis of different parameters of soil before and after sowing of the French bean seeds and the estimation of aerial shoot nitrogen content.

Isolate	Soil					Aerial
	pH	OC (%)	N_2 (%)	P (ppm)	Ph (ppm)	N_2 (%)
Soil (BS)	5.79±0.02	1.21±0.02	0.103±0.003	76.83±0.44	11.67±1.67	--
T0 (AH)	4.94±0.02	1.38±0.02	0.113±0.007	67.83±0.60	18.33±1.67	3.16± 0.03
MTCC99	5.61±0.03	1.58±0.02	0.137±0.003	70.43±0.80	18.33±1.67	4.11± 0.02
DJG	4.80±0.01	1.59±0.01	0.145±0.005	80.33±0.88	16.67±1.67	5.12± 0.02
RSm-3	5.35±0.03	1.59±0.02	0.137±0.004	70.30±0.52	16.67±1.67	4.90± 0.03
KPG-5N	5.17±0.01	1.41±0.01	0.123±0.003	79.33±0.35	18.33±1.67	4.75± 0.02
SKM-N	4.89±0.01	1.48±0.01	0.127±0.003	70.60±0.70	18.33±1.67	4.39± 0.09
RSv-1	5.64±0.03	1.33±0.02	0.121±0.011	72.73±0.24	16.67±1.67	4.04± 0.05
SAM-12	5.10±0.03	1.58±0.02	0.137±0.003	78.23±0.50	18.33±1.67	4.12± 0.03
CBR	5.06±0.03	1.49±0.02	0.135±0.005	79.30±0.46	11.67±1.67	4.19± 0.01
BIJ	5.35±0.02	1.52±0.02	0.130±0.006	76.37±0.48	13.33±1.67	4.06± 0.03
RMa-13	5.08±0.02	1.41±0.02	0.119±0.005	66.80±0.90	18.33±1.67	3.97± 0.04
RGJ	5.39±0.02	1.39±0.02	0.119± .005	71.33±0.58	16.67±1.67	4.12± 0.03

OC= Organic carbon; N_2 (%)= Nitrogen content; P= Potash content; Ph= Phosphorus content; ppm= Parts per million; Soil (BS)= Soil before sowing; T0 (AH)= Control after harvest; ±= Standard error pooled from three independent replicas.

A large variation in inciting nodule formation on the root of the host plant was observed in my study. A statistically highly significant difference was obtained within the nodule numbers in reference to the uninoculated control. The mean nodule number per plant ranged from 19 for isolate SAM-12 to 94 for isolate DJG. No nodules were formed in the uninoculated control treated plant.

The nitrogen content of the aerial plant was found to be the highest in DJG treated plant followed by RSm-3 and KPG-5N (refer table 4.6). Thus, the accumulated plant nitrogen is the indication of specific nodulation by the introduced strains which enhances overall nitrogen fixation (Figueiredo *et al*. 2008) consequently leading to better growth of plant and increase of shoot's dry matter.

In this study, attempt has also been made to correlate the different growth parameters of the plant using SPSS version 21 (Table 4.5). The shoot dry weight was found to be statistically highly correlated with various important growth parameters of plant like the plant height, fresh weight as well as the aerial nitrogen content of the plant.

The nodule number was found to be highly correlated with the nitrogen content. However, the increase in the number of nodules had no correlation with the increase in dry matter of root and shoot. Otieno *et al*. (2007) reported that the increase in nodules number neither translated to shoot and

root dry matter accumulation nor to the grain yield. This fact indicates that the presence of number of nodules do not guarantee the effectiveness of an inoculums (Provorov *et al.* 1994). Some previous researchers suggested that the selection of symbiotic associations should be done on the basis of shoot and root dry weights and hence, the nodule number should not be considered as an appropriate trait for selection of the most effective N_2 fixing *Rhizobium*-legume associations (Hefny *et al*. 2001; Stajkovic *et al*. 2011).

4.8.2.1. Soil analysis

The different parameters of the soil like pH, organic carbon, soil nitrogen, potash and phosphorus content was characterized before the sowing and after harvest (Table 4.6).

A decrease in pH of the soil was noticed after harvest of the plant. However, an increase in the percentage of organic carbon, nitrogen, potash and phosphorus in the soil was found compared to the uninoculated control. From the data, it was revealed that the maximum increase in the organic carbon, soil nitrogen and potash was found from the soil treated with DJG strains.

A highly significant correlation between the soil nitrogen and organic carbon could be found at a *p* value of 0.01. Similarly, the plant nitrogen shared a positive correlation with soil's organic carbon and nitrogen.

Thus, keeping all these things in mind, the DJG strain showed significantly better results in respect of almost all morphological and nodular characters followed by the strains RSm-3 and KPG-5N .

4.9. Diversity study through molecular characterization

4.9.1. *DNA isolation, purification and quantification*

4.9.1.1. DNA isolation

Rhizobium genomic DNA was isolated using the standard protocol of William and Feil (2012) with minor modifications.

Prior to isolation, an efficient lysis is a prerequisite for good yield of total genomic DNA. Chemical dissolution by hot lauryl sulfate (SDS) and enzymes like lysozyme and proteinase K employed better lysis of the cell wall. The copious amount of exopolysaccharides produced by *Rhizobium* was removed by the CTAB extraction which complexes with the polysaccharides retaining the nucleic

acid in the solution. William and Feil (2012) kept the DNA isolation solutions overnight at –20ºC for precipitation, however it was found that non-nucleic acid impurities precipitated more in this way and hence, the DNA isolation solution was incubated at –20ºC for 2 hr.

4.9.1.2. DNA purification

The presence of several impurities produced during the process like cell wall debris, polysaccharides, proteins and RNA acts as a hindrance for the downstream processing and reactions of DNA and therefore needed to be purified.

The phenol:chloroform:isoamyl alcohol (25:24:1) was found to be effective in removing the protein impurities.

TE buffer (1X) was used as DNA storage solution, as EDTA may block the activity of residual nuclease which might somehow contaminate the DNA.

4.9.1.3. DNA quantification and quality check

Quality and quantity of isolated DNA was measured as per standard protocols as mentioned in the materials & method section. The ratios of A_{260} and A_{280} values obtained spectrophotometrically were calculated for each sample. The ratio of all the DNA samples were found to be around 1.8 (refer table 4.7). This showed that the isolated DNA samples were reasonably pure. The concentration of the isolated DNA samples varied from 310-475 ng /µl.

The Agarose gel electrophoresis was performed to determine the intactness of the DNA. Lambda DNA/*Eco*RI/ *Hind*III double digest and 100bp ladder were used for size determination of DNA bands and the size of the bands were found to be approximately around

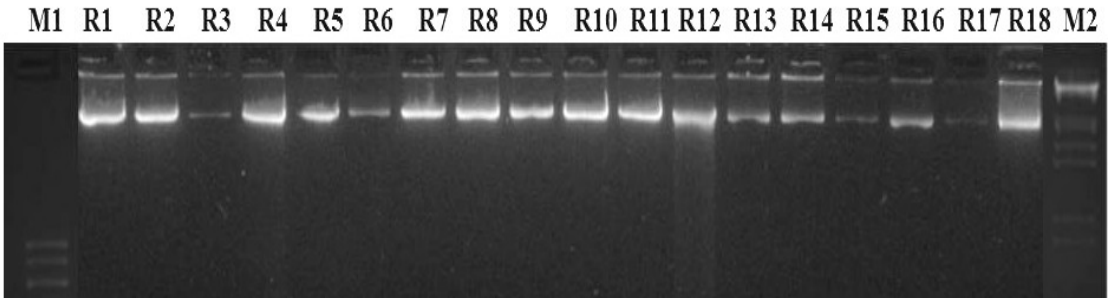

Fig. 4.14. Crude DNA of *Rhizobium.* Lane M1: 100 bp DNA ladder; Lane R1-R18: different *Rhizobium* strains under study (please refer Table 4.7 for the strain's name); Lane M2: λ DNA/ *Eco*RI/ *Hind* III double digest DNA ladder

Table. 4.7. List of *Rhizobium* DNA samples showing their purity

Isolates	Isolate's ID	A_{260}	A_{280}	A_{260}/A_{280} ratio	Quantity of DNA (ng/µl)
MTCC99	R1	0.074	0.041	1.8	370
K-1	R2	0.066	0.036	1.83	330
JPG	R3	0.088	0.048	1.83	440
DJG	R4	0.082	0.047	1.74	410
SKM-N	R5	0.077	0.042	1.83	385
RSm-3	R6	0.062	0.034	1.82	310
CBR	R7	0.092	0.051	1.8	460
KPG-5N	R8	0.083	0.048	1.73	415
BLG	R9	0.066	0.038	1.74	330
BIJ	R10	0.072	0.041	1.76	360
NBU-8	R11	0.095	0.053	1.79	475
MIR-6	R12	0.086	0.046	1.87	430
RSv-1	R13	0.079	0.044	1.79	395
SAM-12	R14	0.069	0.039	1.77	345
RGJ	R15	0.076	0.043	1.77	380
RMa-13	R16	0.087	0.047	1.85	435
SKM-W	R17	0.091	0.05	1.82	455
SKM-E	R18	0.089	0.051	1.75	445

20-21kb (Fig. 4.14).

4.9.2. *RAPD analysis*

RAPD fingerprinting was used for studying the genetic diversity among indigenous *Rhizobium* population. DNA samples from all isolated strains including the reference strain MTCC-99 were tried to amplify with 13 different 10-mer primers. Out of 13 primers, eight primers amplified and produced scorable bands of various intensities. A total of 133 major scorable bands were recorded for phylogenetic analysis. The amplified products ranged in size from about 145 -2200 bp. The amplification patterns revealed 97.74% polymorphism. The number of polymorphic bands generated by each decamer primers ranged in between 12 (OPD-03) and 21 (OPY-04).

Since out of 13 primers only 8 amplified, we made a careful analysis of the sequences of the primers and found that all the primers which failed to amplify were AT-rich. It sounds reasonable now since the *Rhizobium* is largely a GC rich genus.

A representative of RAPD profile photographs of the 18 *Rhizobium* strains generated using OPQ 01, OPA 18 and OPA 02 has been given in Fig. 4.15 A, B & C). Total number and size of amplified bands, number of monomorphic and polymorphic bands and percentage of polymorphism generated by the RAPD primers has been depicted in table 4.8.

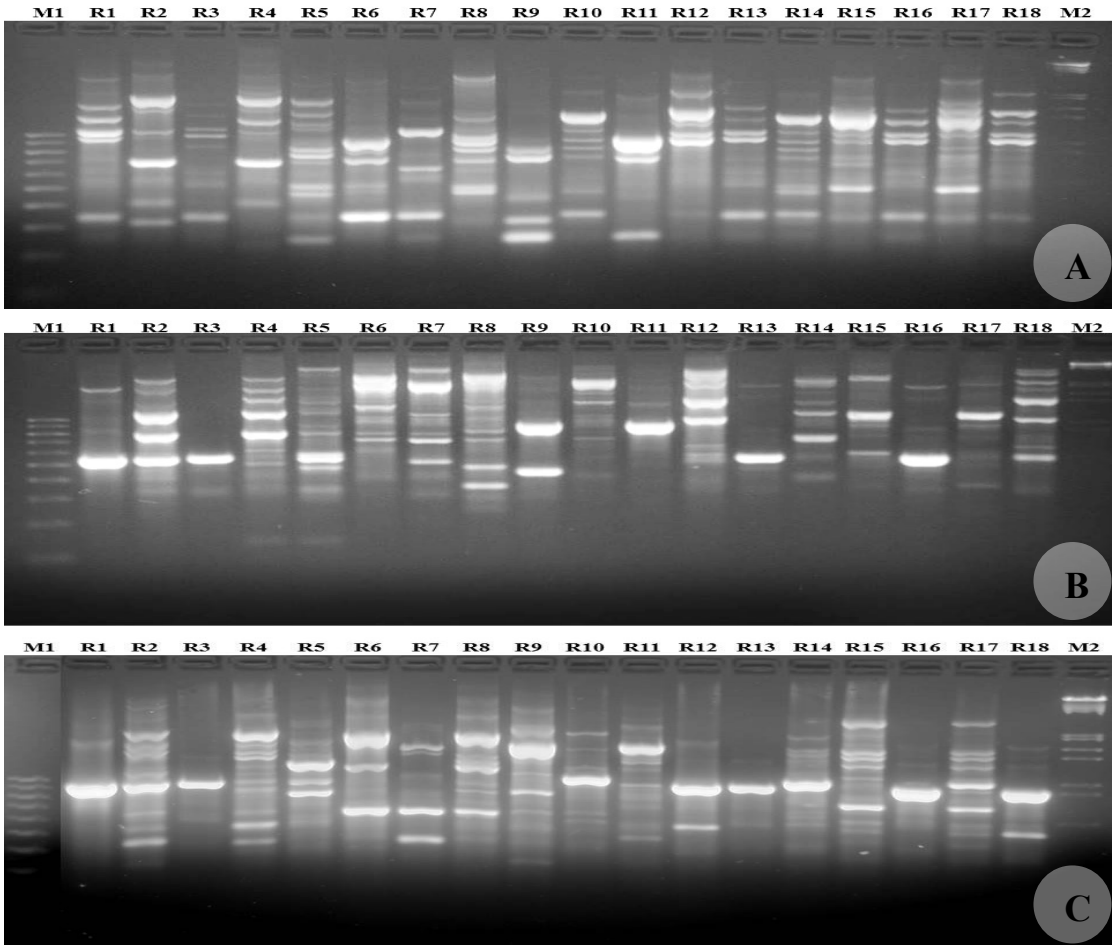

Fig 4.15.Gel showing the DNA bands of eighteen *Rhizobium* strains amplified by the RAPD primers (A)OPQ 01 (B) OPA 18 (C) OPA 02. Lane M1: 100 bp DNA ladder; Lane R1-R18: different *Rhizobium* strains under study (please refer Table 4.7 for the strain's name); Lane M2: λ DNA/ *Eco*RI/ *Hind* III double digest DNA ladder

Table 4.8. Total number and size of amplified bands, number of monomorphic and polymorphic bands and percentage of polymorphism generated by the RAPD primers.

Primer's ID	Sequence (5'-3')	Band No	MB	PB	Pol %	Band size (bp)
OPA 02	TGCCGAGCTG	17	0	17	100%	425-2200
OPD 03	GTCGCCGTCA	12	1	11	92%	378-1792
DAF 9	CCGACGCGGC	16	0	16	100%	157-1034
OPI 06	AAGGCGGCAG	20	0	20	100%	272-1792
OPY 04	AAGGCTCGAC	21	0	21	100%	275-1833
OPQ 01	GGGACGATGG	18	0	18	100%	165-1137
OPA18	AGGTGACCGT	15	0	15	100%	145-1875
CRL 7	GCCCGCCGCC	14	2	12	86%	282-1911
Total		133	3	130	97.74%	

MB= Monomorphic bands; PB= Polymorphic bands; Pol %= Polymorphism %

The similarity matrix obtained using the Dice coefficient of similarity (Nei and Li 1979) has been depicted in table 4.9.

The RAPD profiles of the 18 *Rhizobium* strains were converted into two-dimensional binary matrix and analyzed with NTSYSpc2 programme.

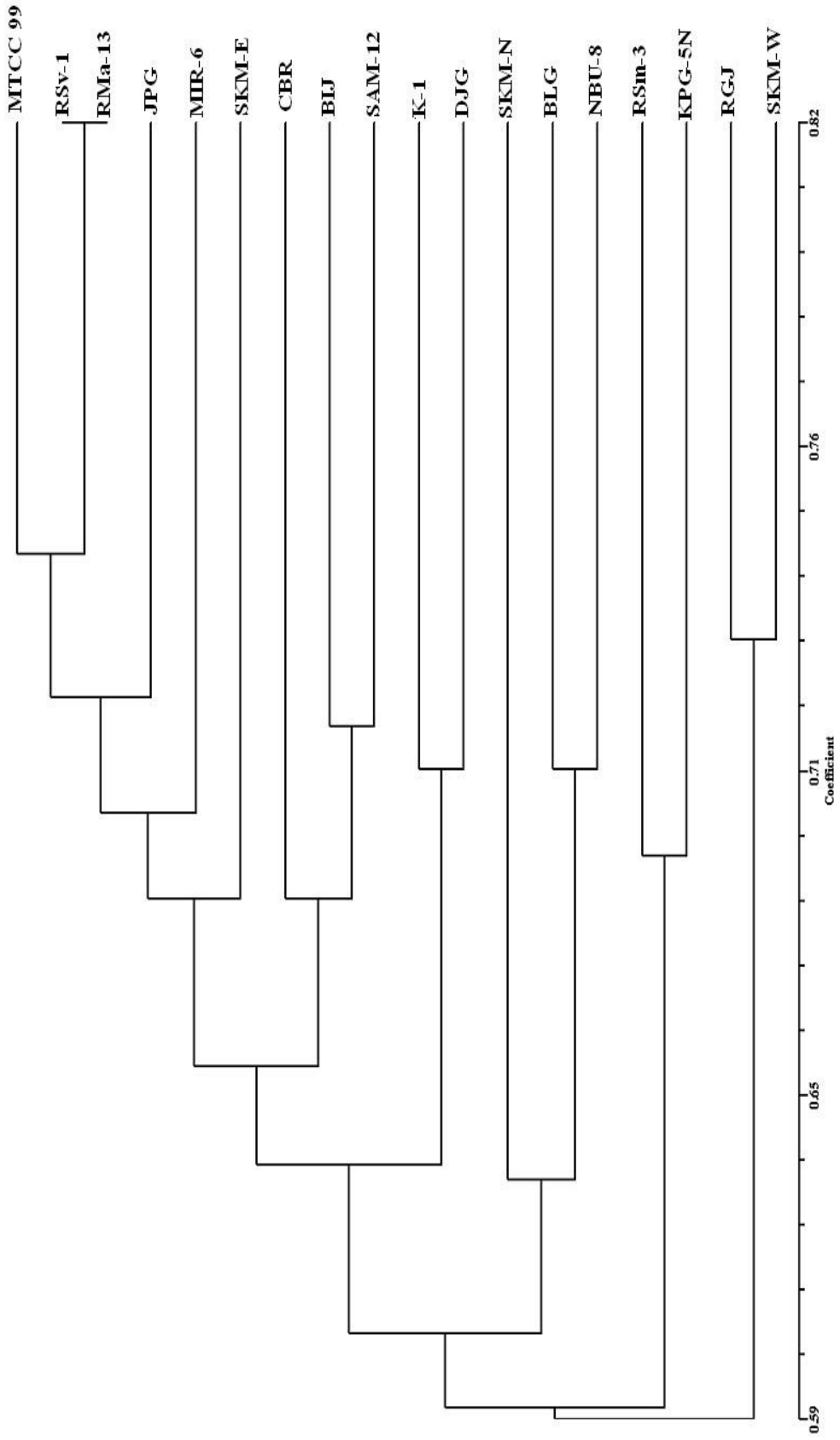

Fig 4.16 Dendrogram derived from UPGMA cluster analysis of RAPD markers illustrating the genetic relationships among the 18 *Rhizobium* strains

Table 4.9. The similarity matrix obtained using Dice coefficient of similarity among the 18 strains of *Rhizobium* based on RAPD profiling

	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15	R16	R17	R18
R1	1.000																	
R2	0.677	1.000																
R3	0.684	0.677	1.000															
R4	0.684	0.707	0.579	1.000														
R5	0.654	0.617	0.684	0.654	1.000													
R6	0.624	0.632	0.564	0.594	0.549	1.000												
R7	0.662	0.684	0.677	0.632	0.617	0.632	1.000											
R8	0.632	0.609	0.556	0.617	0.647	0.692	0.504	1.000										
R9	0.617	0.594	0.586	0.602	0.647	0.556	0.519	0.654	1.000									
R10	0.692	0.594	0.617	0.677	0.602	0.662	0.684	0.579	0.549	1.000								
R11	0.609	0.617	0.669	0.624	0.624	0.534	0.602	0.556	0.707	0.617	1.000							
R12	0.707	0.669	0.647	0.692	0.662	0.586	0.609	0.609	0.624	0.669	0.617	1.000						
R13	0.722	0.624	0.767	0.617	0.677	0.556	0.669	0.609	0.639	0.714	0.647	0.774	1.000					
R14	0.617	0.564	0.556	0.586	0.556	0.617	0.684	0.624	0.579	0.714	0.571	0.654	0.699	1.000				
R15	0.609	0.571	0.549	0.594	0.564	0.579	0.586	0.617	0.586	0.617	0.654	0.617	0.617	0.662	1.000			
R16	0.767	0.594	0.707	0.662	0.617	0.556	0.654	0.624	0.564	0.684	0.586	0.684	0.820	0.639	0.632	1.000		
R17	0.669	0.586	0.624	0.564	0.534	0.579	0.541	0.602	0.496	0.571	0.579	0.586	0.586	0.586	0.729	0.662	1.000	
R18	0.669	0.586	0.624	0.699	0.564	0.594	0.677	0.617	0.556	0.662	0.609	0.707	0.707	0.662	0.609	0.722	0.564	1.000

For details on sample ID R1 to R18 please refer Table 4.7

The resulting dendrogram, generated by using eight oligonucleotide primers has been shown in Fig. 4.16.

The highest similarity (82%) was found between RSv-1 and RMa-13 strains. The similarity matrix data revealed that the phylogenetic relationship between RSv-1 and RMa-13 was closest, followed by the reference strain MTCC-99. The lowest level of similarity existed between two strains BLG and SKM-W(49.65%) .

However, no proper clustering of the strains based on the RAPD generated fingerprints could be obtained. Loosely three clusters could be figured out from the dendrogram. Cluster I consisted of five strains RSv-1, RMa-13, JPG, MIR-6, SKM-E including the reference MTCC-99 strain. Cluster II consisted of CBR, BIJ and SAM, while Cluster III comprised of SKM-N, BLG and NBU-8 strains. However, strains like K-1, DGJ, RSm-3, KPG-5N, RGJ and SKM-W remained as a pair. The isolates were found to be genetically very diverse as no cluster was formed according to their geographical location.

4.9.3. *rep- PCR based fingerprinting*

rep-PCR amplification with REP, ERIC and BOX primers were performed to produce DNA fingerprints with reproducible and specific banding patterns. The details of the total number and size of amplified bands, number of monomorphic and polymorphic bands and percentage of polymorphism generated by the rep-PCR primers has been elucidated in table 4.10.

4.9.3.1. *Repetitive Extragenic Palindromic (REP)–PCR*

Informative banding patterns were obtained for all strains analyzed by using REP-primers (Fig 4.17). The REP primer set yielded a total of 27 fragments that were used for the construction of dendrogram. The primers revealed 100% polymorphism as no single monomorphic band was observed. The band size of the DNA fragments was found to be between

Table 4.10. Total number and size of amplified bands, number of monomorphic and polymorphic bands and percentage of polymorphism generated by the rep-PCR primers

rep-PCR	Band No	MB	PB	Pol(%)	Band size (bp)
REP-PCR	27	0	27	100	2.153-20,000
ERIC-PCR	29	1	28	96.55	1.072-20,778
BOX-PCR	31	2	29	93.54	2.201-16,167
Total	**87**	**3**	**84**	**96.55**	

MB= Monomorphic bands; PB= Polymorphic bands; Pol %= Polymorphism %

0.21-2.0 kb. Sikora *et al*. (2002) also obtained similar band size with REP primers.

The similarity coefficient of the isolates ranged from 0.333-0.926 (Table 4.11). The two strains SKM-N isolated from North Sikkim and RSm-3 isolated from Sonada of North Bengal shared the maximum similarity while the lowest similarity was found between the isolates BLG of Balurghat and SKM-E of East Sikkim.

However, clustering of strains as per geographic locations ended here. In the other parts of the dendogram, strains were not flocked based on location-wise closeness (Fig. 4.18).

4.9.3.2. *ERIC –PCR*

The ERIC (Enterobacterial repetitive intergenic consensus sequences) primer set (ERIC IR and ERIC 2, forward and reverse respectively) produced altogether 29 bands out of which 28 were polymorphic revealing 96.55% polymorphism. The band size of the DNA fragments was found to be between 0.11-2.0 kb similar to REP-PCR (Fig. 4.19).

The similarity coefficient of the strains ranged from 0.483 to 0.862 (Table

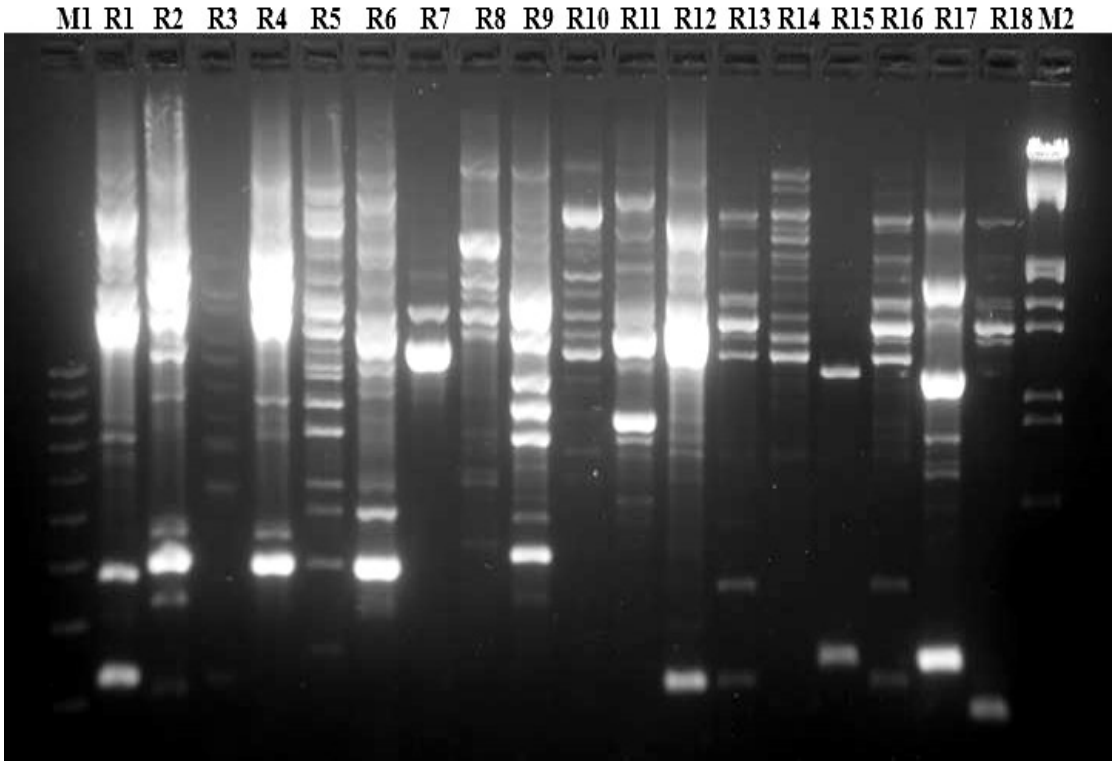

Fig.4.17 Gel showing the DNA bands of eighteen *Rhizobium* strains amplified by REP primer. Lane M1: 100 bp DNA ladder; Lane R1-R18: different *Rhizobium* strains under study (please refer Table 4.7 for the strain's name); Lane M2: λ DNA/ *Eco*RI/ *Hind* III double digest DNA ladder

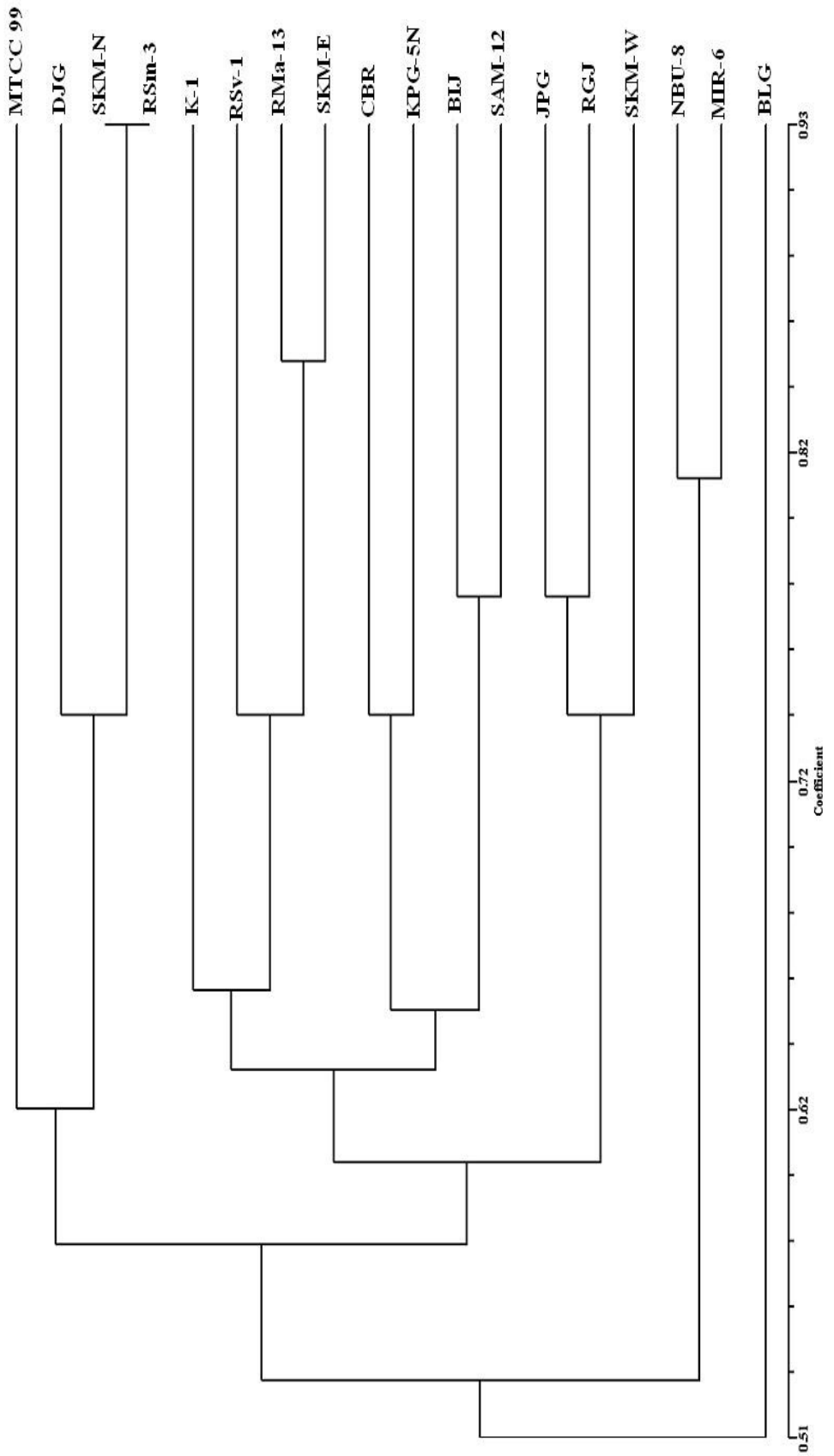

Fig. 4.18. Dendrogram derived from UPGMA cluster analysis generated by REP-PCR primer illustrating the genetic relationship among 18 *Rhizobium* strains.

Table 4.11. The similarity matrix obtained using Dice coefficient of similarity among the 18 accessions of *Rhizobium* based on REP-PCR profiling

	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15	R16	R17	R18
R1	1.000																	
R2	0.593	1.000																
R3	0.630	0.444	1.000															
R4	0.741	0.556	0.667	1.000														
R5	0.593	0.556	0.593	0.778	1.000													
R6	0.519	0.556	0.519	0.704	0.926	1.000												
R7	0.407	0.667	0.630	0.519	0.593	0.667	1.000											
R8	0.519	0.556	0.667	0.630	0.704	0.704	0.741	1.000										
R9	0.481	0.519	0.407	0.519	0.593	0.593	0.556	0.667	1.000									
R10	0.593	0.481	0.519	0.556	0.556	0.556	0.667	0.630	0.667	1.000								
R11	0.556	0.444	0.630	0.444	0.444	0.519	0.556	0.593	0.481	0.593	1.000							
R12	0.593	0.481	0.593	0.407	0.333	0.407	0.667	0.556	0.519	0.630	0.815	1.000						
R13	0.593	0.704	0.519	0.556	0.630	0.630	0.741	0.556	0.519	0.630	0.444	0.630	1.000					
R14	0.593	0.630	0.519	0.630	0.704	0.630	0.667	0.630	0.593	0.778	0.444	0.481	0.704	1.000				
R15	0.556	0.593	0.778	0.519	0.444	0.519	0.778	0.593	0.407	0.519	0.630	0.667	0.667	0.519	1.000			
R16	0.630	0.667	0.630	0.593	0.593	0.519	0.704	0.593	0.407	0.593	0.481	0.667	0.815	0.667	0.630	1.000		
R17	0.630	0.593	0.778	0.519	0.519	0.444	0.556	0.593	0.481	0.519	0.481	0.519	0.593	0.593	0.704	0.630	1.000	
R18	0.630	0.593	0.704	0.593	0.519	0.444	0.704	0.593	0.333	0.593	0.481	0.593	0.667	0.667	0.704	0.852	0.704	1.000

For details on sample ID R1 to R18 please refer Table 4.7

4.12). The highest similarity level was shared by the isolates RMa-13 of North Bengal and SKM-W of West Sikkim. The lowest genetic similarity (0.483) was found between the isolates DJG with SAM-12 and SKM-W.

The NTSys based dendrogram of ERIC –PCR represented the formation of three clusters. Most of the studied strains were grouped with the first major cluster (cluster I) largely ignoring their place of origin. The eight strains in the cluster I differed at a similarity level of 0.68 from the other strains. However, two sub-clusters may be derived from Cluster I. The upper sub-cluster consisted of MTCC99, JPG, CBR and KPG-5N while the other sub-cluster contained the strains K-1, DJG, RSm-3 and NBU-8. The cluster II consisted of three strains while cluster III consisted of seven strains. All the strains in the clusters have grouped together irrespective of their isolation sites (Fig. 4.20).

4.9.3.3. *BOX-PCR*

The BOX primer produced a total of 31 bands revealing 93.54% polymorphism. The band size of the DNA fragments was found to be 0.22–1.6 kb (Fig. 4.21).

The similarity coefficient of the isolates ranged from 0.484 to 1.000

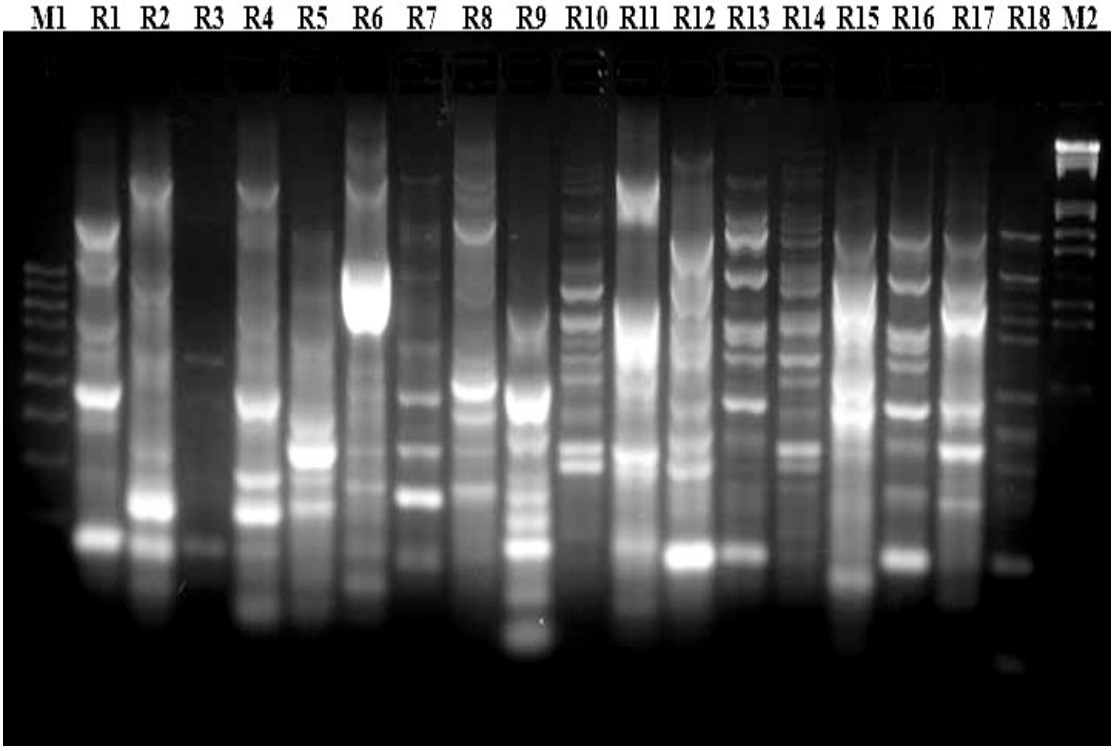

Fig.4.19 Gel showing the DNA bands of eighteen *Rhizobium* strains amplified by ERIC primer. Lane M1: 100 bp DNA ladder; Lane R1-R18: different *Rhizobium* strains under study (please refer Table 4.7 for the strain's name); Lane M2: λ DNA/ *Eco*RI/ *Hind* III double digest DNA ladder

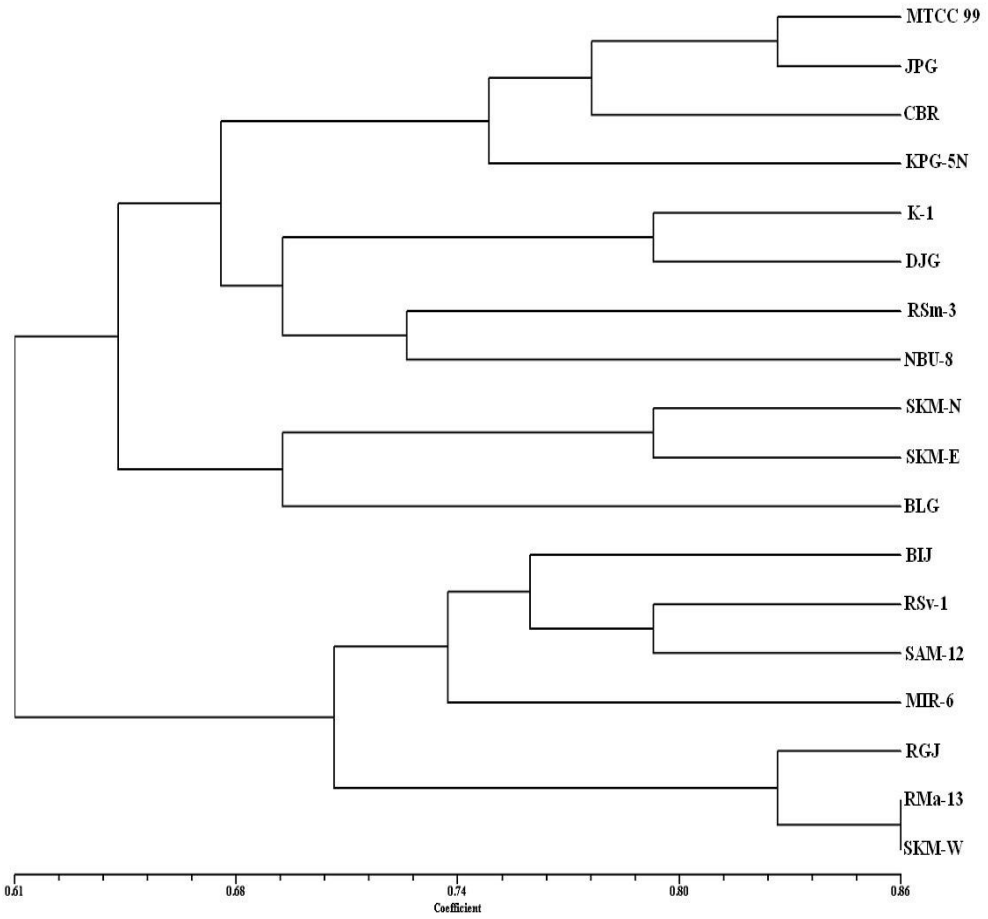

Fig. 4.20. Dendrogram derived from UPGMA cluster analysis generated by ERIC-PCR primer illustrating the genetic relationship among 18 *Rhizobium* strains.

(table 4.13). The strains JPG and RMa-13 was found to be the closest with 100% similarity level. Such 100% similarity level was also observed between the isolates of Sikkim origin i.e. SKM-E and SKM-W. The lowest level of similarity (48.4%) existed between two strains NBU-8 and the reference strain MTCC -99.

On the basis of the dendrogram generated by NTSYSpc-2, three clusters could be obtained at a node of 0.75. Cluster I consisted of five strains K-1, RGJ, CBR, SAM-12 and BIJ while cluster II contained four strains namely, JPG, RMa-13, RSv-1 and MIR-6. The two clusters diverged at a similarity level of 0.77. The two Sikkim (SKM-W and SKM-E) strains and DJG grouped to form the third cluster. The hill strain RSm-3 diverged at a level of 0.64 from other strains clubbing with the reference MTCC-99 strain (Fig. 4.22).

4.9.3.4. *Combined rep-PCR analysis*

Each rep-PCR (REP-PCR, ERIC-PCR

Table 4.12. The similarity matrix obtained using Dice coefficient of similarity among the 18 accessions of *Rhizobium* based on ERIC-PCR profiling

	Rh1	Rh2	Rh3	Rh4	Rh5	Rh6	Rh7	Rh8	Rh9	Rh10	Rh11	Rh12	Rh13	Rh14	Rh15	Rh16	Rh17	Rh18
Rh1	1.000																	
Rh2	0.586	1.000																
Rh3	0.828	0.759	1.000															
Rh4	0.724	0.793	0.759	1.000														
Rh5	0.724	0.655	0.690	0.655	1.000													
Rh6	0.724	0.655	0.621	0.724	0.724	1.000												
Rh7	0.759	0.621	0.793	0.621	0.759	0.621	1.000											
Rh8	0.793	0.655	0.690	0.655	0.655	0.655	0.759	1.000										
Rh9	0.690	0.621	0.724	0.621	0.690	0.621	0.586	0.552	1.000									
Rh10	0.655	0.724	0.621	0.586	0.655	0.517	0.552	0.724	0.621	1.000								
Rh11	0.724	0.655	0.759	0.724	0.586	0.724	0.621	0.655	0.690	0.655	1.000							
Rh12	0.690	0.690	0.655	0.621	0.690	0.552	0.586	0.621	0.724	0.759	0.621	1.000						
Rh13	0.690	0.621	0.655	0.552	0.621	0.483	0.655	0.690	0.655	0.759	0.552	0.724	1.000					
Rh14	0.621	0.621	0.586	0.483	0.552	0.483	0.517	0.621	0.586	0.759	0.621	0.724	0.793	1.000				
Rh15	0.655	0.655	0.690	0.655	0.724	0.655	0.621	0.655	0.621	0.655	0.586	0.690	0.690	0.690	1.000			
Rh16	0.621	0.621	0.655	0.552	0.759	0.621	0.655	0.552	0.586	0.621	0.552	0.724	0.724	0.724	0.828	1.000		
Rh17	0.552	0.552	0.586	0.483	0.759	0.483	0.655	0.621	0.517	0.690	0.483	0.655	0.793	0.793	0.828	0.862	1.000	
Rh18	0.586	0.655	0.621	0.655	0.793	0.586	0.690	0.517	0.690	0.586	0.586	0.690	0.621	0.552	0.586	0.690	0.621	1.000

For details on sample ID R1 to R18 please refer Table 4.7

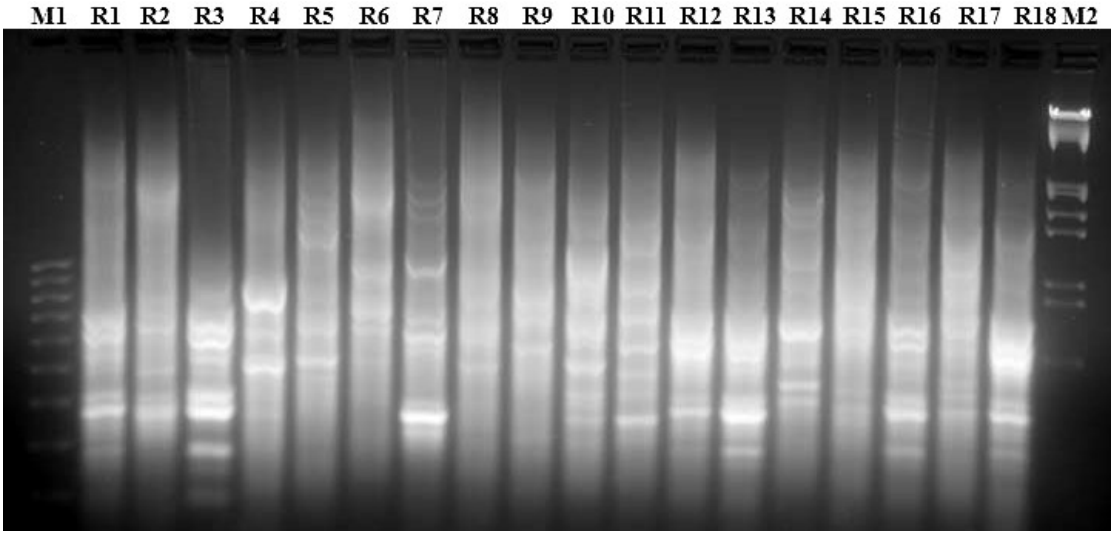

Fig.4.21 Gel showing the DNA bands of eighteen *Rhizobium* strains amplified by BOX primer. Lane M1: 100 bp DNA ladder; Lane R1-R18: different *Rhizobium* strains under study (please refer Table 4.7 for the strain's name); Lane M2: λ DNA/ *Eco*RI/ *Hind* III double digest DNA ladder

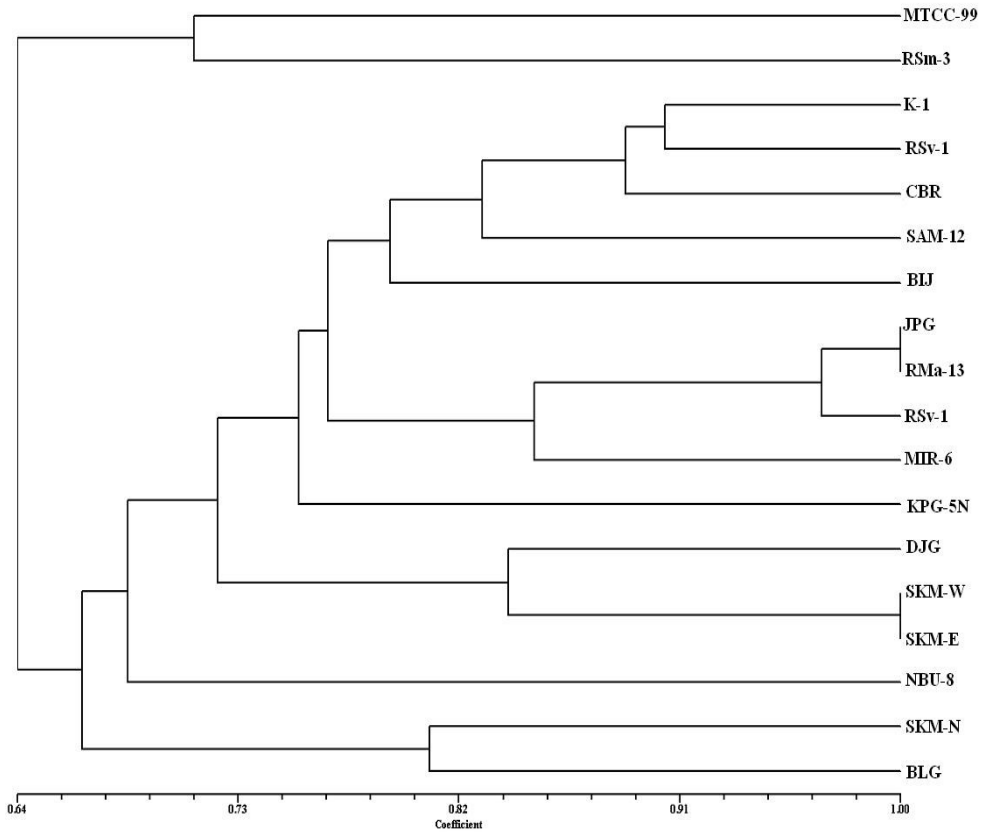

Fig. 4.22. Dendrogram derived from UPGMA cluster analysis generated by BOX-PCR primer illustrating the genetic relationship among 18 *Rhizobium* strains.

and BOX-PCR) pattern generated a unique fingerprint and no similarity was observed in clustering of the isolates. The number of matrices (29 in REP-PCR, 27 in ERIC-PCR and 31 in BOX-PCR and variations in the rep-PCR patterns revealing 96.55% polymorphism were so large that the

Table 4.13. The similarity matrix obtained using Dice coefficient of similarity among the 18 accessions of *Rhizobium* based on BOX-PCR profiling

	Rh1	Rh2	Rh3	Rh4	Rh5	Rh6	Rh7	Rh8	Rh9	Rh10	Rh11	Rh12	Rh13	Rh14	Rh15	Rh16	Rh17	Rh18
Rh1	1.000																	
Rh2	0.677	1.000																
Rh3	0.710	0.774	1.000															
Rh4	0.581	0.581	0.677	1.000														
Rh5	0.581	0.581	0.677	0.613	1.000													
Rh6	0.710	0.645	0.613	0.548	0.548	1.000												
Rh7	0.677	0.871	0.839	0.645	0.645	0.645	1.000											
Rh8	0.742	0.806	0.774	0.581	0.581	0.645	0.742	1.000										
Rh9	0.645	0.581	0.742	0.548	0.806	0.613	0.645	0.710	1.000									
Rh10	0.710	0.774	0.742	0.613	0.613	0.677	0.774	0.774	0.613	1.000								
Rh11	0.484	0.742	0.645	0.581	0.581	0.645	0.677	0.677	0.645	0.710	1.000							
Rh12	0.677	0.677	0.839	0.710	0.774	0.710	0.742	0.677	0.839	0.710	0.742	1.000						
Rh13	0.677	0.806	0.968	0.645	0.710	0.645	0.871	0.742	0.774	0.710	0.677	0.871	1.000					
Rh14	0.581	0.774	0.742	0.742	0.613	0.613	0.839	0.710	0.548	0.806	0.710	0.710	0.710	1.000				
Rh15	0.645	0.903	0.806	0.677	0.677	0.613	0.903	0.774	0.613	0.806	0.710	0.710	0.839	0.871	1.000			
Rh16	0.710	0.774	1.000	0.677	0.677	0.613	0.839	0.774	0.742	0.742	0.645	0.839	0.968	0.742	0.806	1.000		
Rh17	0.613	0.742	0.710	0.839	0.774	0.645	0.806	0.613	0.645	0.710	0.677	0.806	0.742	0.839	0.839	0.710	1.000	
Rh18	0.613	0.742	0.710	0.839	0.774	0.645	0.806	0.613	0.645	0.710	0.677	0.806	0.742	0.839	0.839	0.710	1.000	1.000

For details on sample ID R1 to R18 please refer Table 4.7

proper cluster analysis of the strains could not be obtained. Hence, inorder to obtain a more detailed cluster analysis, the matrices generated by REP, ERIC and BOX-PCR were combined and a dendrogram was generated by NTSys. The cluster analysis at a node of 0.70 level showed the formation of only two loose clusters (Fig 4.23).

Cluster I consisted of JPG, CBR, RSv-1 and RMa-13 while cluster II consisted of RGJ, SKM-W and SKM-E. However, the other strains occurred in singlet or in doublet. Phylogenetically the closest distance existed between RSv-1 and RMa-13 (83.9%) while RSm-3 and SKM-W shared a lowest genetic distance (Table 4.14). The combined rep-PCR result also showed the strains to cluster irrespective of their geographical location revealing a high level of genetic diversity. Pinto *et al*. (2007)

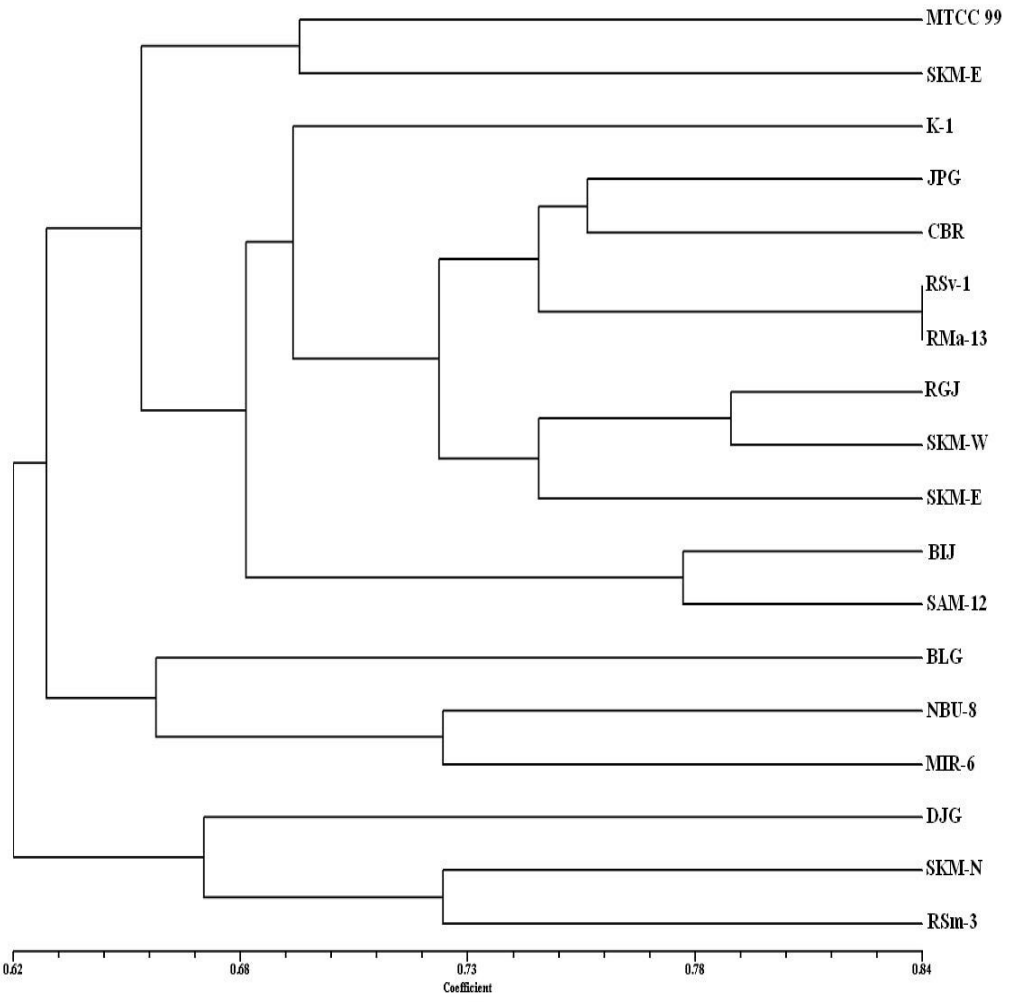

Fig. 4.23. Dendrogram derived from UPGMA cluster analysis generated by combined rep-PCR(REP, ERIC and BOX) illustrating the genetic relationship among 18 *Rhizobium* strains.

Table 4.14. The similarity matrix obtained using Dice coefficient of similarity among the 18 accessions of *Rhizobium* based on combined rep-PCR profiling

	Rh1	Rh2	Rh3	Rh4	Rh5	Rh6	Rh7	Rh8	Rh9	Rh10	Rh11	Rh12	Rh13	Rh14	Rh15	Rh16	Rh17	Rh18
Rh1	1.000																	
Rh2	0.621	1.000																
Rh3	0.724	0.667	1.000															
Rh4	0.678	0.644	0.701	1.000														
Rh5	0.632	0.598	0.655	0.678	1.000													
Rh6	0.655	0.621	0.586	0.655	0.724	1.000												
Rh7	0.621	0.724	0.759	0.598	0.667	0.644	1.000											
Rh8	0.690	0.678	0.713	0.621	0.644	0.667	0.747	1.000										
Rh9	0.609	0.575	0.632	0.563	0.701	0.609	0.598	0.644	1.000									
Rh10	0.655	0.667	0.632	0.586	0.609	0.586	0.667	0.713	0.632	1.000								
Rh11	0.586	0.621	0.678	0.586	0.540	0.632	0.621	0.644	0.609	0.655	1.000							
Rh12	0.655	0.621	0.701	0.586	0.609	0.563	0.667	0.621	0.701	0.701	0.724	1.000						
Rh13	0.655	0.713	0.724	0.586	0.655	0.586	0.759	0.667	0.655	0.701	0.563	0.747	1.000					
Rh14	0.598	0.678	0.621	0.621	0.621	0.575	0.678	0.655	0.575	0.782	0.598	0.644	0.736	1.000				
Rh15	0.621	0.724	0.759	0.621	0.621	0.598	0.770	0.678	0.552	0.667	0.644	0.690	0.736	0.701	1.000			
Rh16	0.655	0.690	0.770	0.609	0.678	0.586	0.736	0.644	0.586	0.655	0.563	0.747	0.839	0.713	0.759	1.000		
Rh17	0.598	0.632	0.690	0.621	0.690	0.529	0.678	0.609	0.552	0.644	0.552	0.667	0.713	0.747	0.793	0.736	1.000	
Rh18	0.609	0.667	0.678	0.701	0.701	0.563	0.736	0.575	0.563	0.632	0.586	0.701	0.678	0.690	0.713	0.747	0.782	1.000

For details on sample ID R1 to R18 please refer Table 4.7

also observed similar result with the combined rep-PCR analysis of Brazilian *R. tropici* strains.

4.9.4. *ARDRA or PCR-RFLP*

Amplified Ribosomal Restriction DNA Analysis (ARDRA), an extension of RFLP involves an enzymatic amplification of the conserved 16SrRNA gene, followed by digestion with the restriction enzymes. PCR-RFLP being a simple yet inexpensive method, widely used to phylogenetically characterize and assess the genetic diversity of different *Rhizobium* isolates. Thus, it was applied to study the fingerprinting of different strains of *Rhizobium* found in the studied area.

4.9.4.1. *16SrDNA–PCR amplification*

The partial conserved 16SrRNA genes of all 18 French bean rhizobial isoates were subjected to amplification with universal ribosomal primers 337F and 907R. The primer pair successfully amplified the conserved region of the genome in all the isolates.

The size of the amplified products were determined through Gel electrophoresis using PhotoCapt software and were found to be 554-619 bp (Fig. 4.24).

4.9.4.2. *16SrDNA PCR product restriction digestion*

We used a total of 8 restriction enzymes (a mixed bag of both rare cutter 6-base cutter and frequent cutter (4-base cutter) restriction enzymes (REs) (refer M&M for list of Restriction enzymes). The bands produced by the restriction digestion with the enzymes *Alu* I, *Hpa*II and *Mbo* I has been given in Fig.4.25. The total fragments, number of monomorphic and polymorphic bands of 16SrDNA generated using different restriction

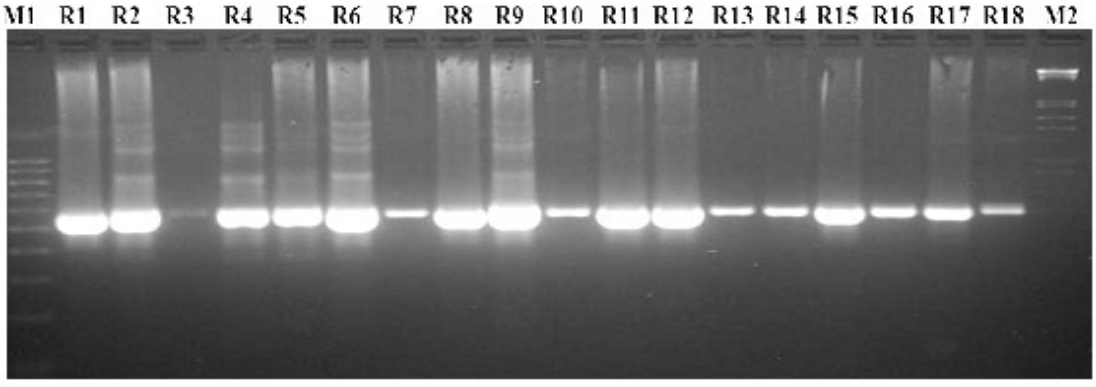

Fig 4.24. Amplification of partial *Rhizobium* DNA with 16SrDNA primers (337F and 907R) . Lane M1: 100 bp DNA ladder; Lane R1-R18: different *Rhizobium* strains under study (please refer Table 4.7 for the strain's name); Lane M2: λ DNA/ *Eco*RI/ *Hind* III double digest DNA ladder

enzymes has been elucidated in table 4.15.

Except *Pst*I all the REs used produced reproducible bands. A total of 42 scorable bands with the band size ranging from 60-982 bp were generated. The polymorphism percentage was found to be 64.28%. The highest (83.3%) polymorphism was recorded with *Hinf* I while the lowest (25%) polymorphism was observed with *Eco*RI. The similarity coefficient value ranged from 50-100% (table 4.16).

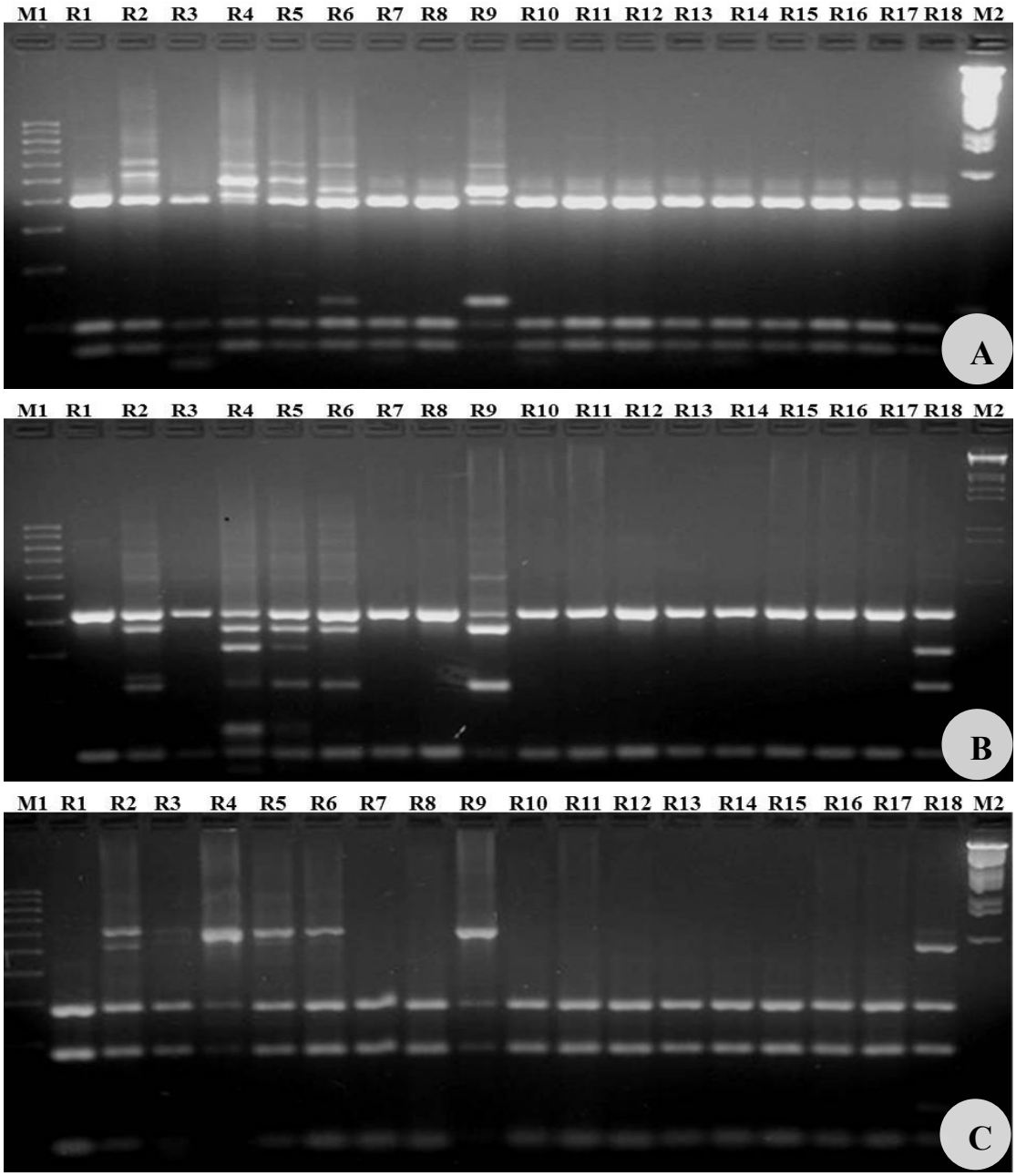

Fig.4.25 Restriction digestion products of partial region of 16s rDNA (A)AluI (B) Hpa II (C) MboI. Lane M1: 100 bp DNA ladder; Lane R1-R18: different *Rhizobium* strains under study (please refer Table 4.7 for the strain's name); Lane M2: λ DNA/ *Eco*RI/ *Hind* III double digest DNA ladder

Table 4.15. Total fragments, number of monomorphic and polymorphic bands of 16SrDNA generated using different restriction enzymes

Restriction enzymes	No. of cuts	MB	PB	Pol%	Band size
Alu I	7	3	4	57.14	60-621
Hpa II	8	2	6	75	88-592
Taq 1	6	2	4	66.6	170-610
Mbo I	5	2	3	60	105-646
*Eco*RI	4	3	1	25	242-592
*Hae*III	6	2	4	66.6	47-579
Hinf I	6	1	5	83.3	200-982
Total 42		**15**	**27**	**64.28**	

MB= Monomorphic bands; PB= Polymorphic bands; Pol%= Polymorphism %

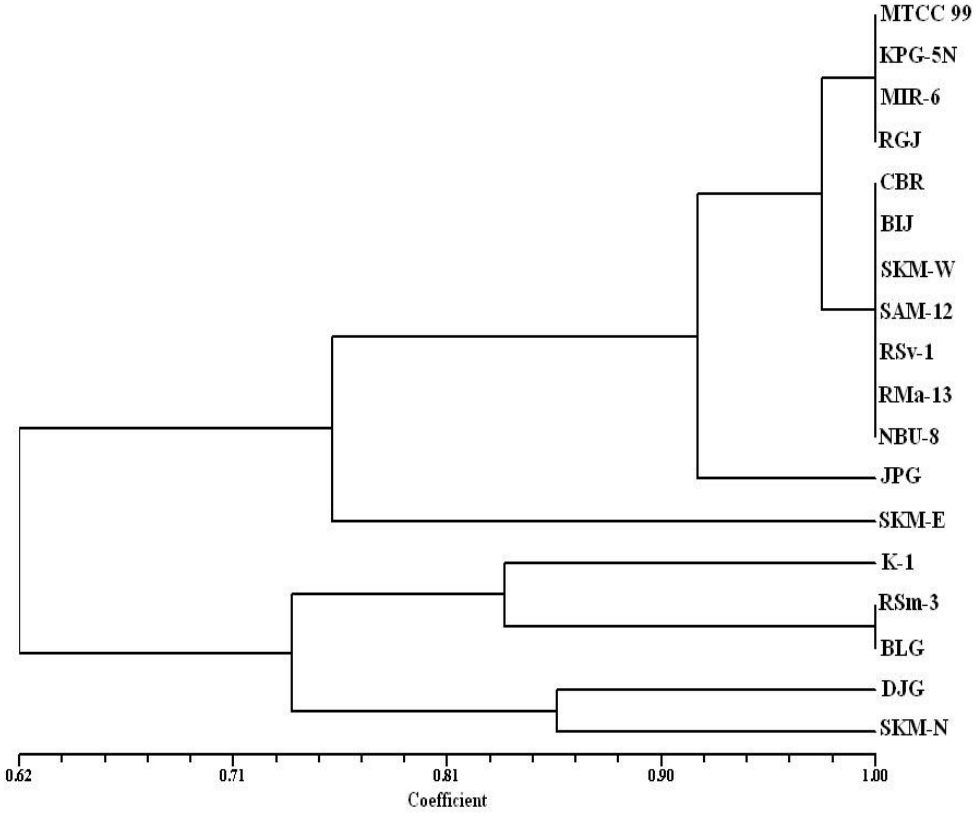

Fig 4.26. Dendrogram based on the restriction digestion products data of the partial region of 16s rDNA

A dendrogram was generated from the distance matrix data of 16SrDNA-PCR RFLP fingerprints by NTSYSpc-2. A major division at 0.73 separated the strains into two clusters (cluster I and II). Out of the eighteen strains, 13 *Rhizobium* strains were grouped with the first major cluster along with the reference strain MTCC-99 (Fig.4.26). The 13 strains consisting cluster I were MTCC-99, KPG-5N, MIR-6, RGJ, CBR, BIJ, SKM-W, SAM-12, RSv-1, RMa-13, NBU-8, JPG and SKM-E while cluster II consisted of K-1, RSm-

Table 4.16. The similarity matrix obtained using Dice coefficient of similarity among the 18 accessions of *Rhizobium* based on 16SrDNA-PCR-RFLP profiling

	Rh1	Rh2	Rh3	Rh4	Rh5	Rh6	Rh7	Rh8	Rh9	Rh10	Rh11	Rh12	Rh13	Rh14	Rh15	Rh16	Rh17	Rh18
Rh1	1.000																	
Rh2	0.650	1.000																
Rh3	0.875	0.625	1.000															
Rh4	0.625	0.825	0.600	1.000														
Rh5	0.575	0.825	0.550	0.950	1.000													
Rh6	0.700	0.850	0.675	0.825	0.775	1.000												
Rh7	1.000	0.650	0.875	0.625	0.575	0.700	1.000											
Rh8	1.000	0.650	0.875	0.625	0.575	0.700	1.000	1.000										
Rh9	0.700	0.850	0.675	0.825	0.775	1.000	0.700	0.700	1.000									
Rh10	0.975	0.625	0.900	0.600	0.550	0.675	0.975	0.975	0.675	1.000								
Rh11	1.000	0.650	0.875	0.625	0.575	0.700	1.000	1.000	0.700	0.975	1.000							
Rh12	1.000	0.650	0.875	0.625	0.575	0.700	1.000	1.000	0.700	0.975	1.000	1.000						
Rh13	1.000	0.650	0.875	0.625	0.575	0.700	1.000	1.000	0.700	0.975	1.000	1.000	1.000					
Rh14	0.975	0.625	0.900	0.600	0.550	0.675	0.975	0.975	0.675	1.000	0.975	0.975	0.975	1.000				
Rh15	1.000	0.650	0.875	0.625	0.575	0.700	1.000	1.000	0.700	0.975	1.000	1.000	1.000	0.975	1.000			
Rh16	1.000	0.650	0.875	0.625	0.575	0.700	1.000	1.000	0.700	0.975	1.000	1.000	1.000	0.975	1.000	1.000		
Rh17	1.000	0.650	0.875	0.625	0.575	0.700	1.000	1.000	0.700	0.975	1.000	1.000	1.000	0.975	1.000	1.000	1.000	
Rh18	0.825	0.675	0.700	0.650	0.650	0.675	0.825	0.825	0.675	0.800	0.825	0.825	0.825	0.800	0.825	0.825	0.825	1.000

For details on sample ID R1 to R18 please refer Table 4.7

3, BLG, DJG and SKM-N. The clustering of strains in two distinct separate groups definitely gave an indication of the genetic variation among the strains. However, similar to other DNA fingerprinting studies, the PCR-RFLP analysis also revealed the cluster formation of the 18 isolates irrespective of their geographical location.

4.9.5. *Comparative account of DNA fingerprinting studies*

Since many of the popular fingerprinting techniques were not tried in *Rhizobium* and virtually no work has been done on the *Rhizobium* strains found in North Bengal and Sikkim, various fingerprinting techniques were performed to study the native *Rhizobium* strains as well as to make a comparative account of various techniques.

One thing is common from the result that genetic diversity depicted through DNA fingerprinting is independent of altitude of the places where they are found. It was found that strains of Sikkim and Sub-Himalayan regions often clustered with strains of Mahananda and Gangetic plains. Maximum number of bands (133) was found in RAPD as well as maximum number of polymorphism (97.74%) was also detected in RAPD. Bands were also more or less reproducible. Combination of various rep-PCR (REP, ERIC and BOX) also produced good result with distinct studies. Both the markers have been found to provide a cheap, rapid and effective means to evaluate the genetic diversity among the *Rhizobium* isolates. Hence, since both the two DNA markers are useful in their own way, both the DNA fingerprinting studies can be conducted to analyze the genetic diversity among the isolates.

On the other hand, PCR-RFLP of the conserved region of 16SrDNA performed with seven restriction enzymes revealed a polymorphism of 64.28 % which was less compared to RAPD and rep-PCR. Thus it may be concluded that the PCR-RFLP of partial 16SrDNA region of the genome may not be a good option for the study of *Rhizobium* diversity.

4.10. *16S rDNA partial nucleotide sequence parity and phylogenetic analysis*

Out of 18 studied strains, eleven were chosen for partial sequencing of 16SrDNA. Emphasis was given on diversity of strains while choosing for

sequencing.

All the products were sequenced properly and sizes of the sequences were around 500bp. The sequences were subjected to alignment with global partial 16SrDNA database from GenBank through BLAST program.

The result showed that 4 out of eleven strains showed maximum resemblance with various strains of *R. leguminosarum* while three strains were found to be close with strains of *R. etli*. One strain each showed 100% resemblance with *R. phaseoli* and *R. paranense*. Other strains showed maximum closeness with unspecified *Rhizobium* strains (table 4.17).These eleven partial sequences of 16SrDNA along with 34 partial sequences of rhizobial 16SrDNA were used for the construction of phylogenetic tree keeping *Rhodobacter sphaeroides* of family Rhodobacteriaceae as out-group.

The dendrogram was generated by RAxML on the basis of maximum likelihood built with 1000 bootstrap value using the PROTCAT_GAMMAI WAG substitution model. From the phylogeny tree it was found that the closest neighbor of *R. sphaeroides* are *Azorhizobium* and *Bradyrhizobium* followed by strains of *Mesorhizobium* and *Sinorhizobium* etc.

Table 4.17. GenBank accession numbers for partial 16SrRNA sequence of *Rhizobium* strains

Strains	**Isolates (location)**	**Se-quence's Length (bp)**	**GenBank Accession No.**	**Maximum similarity(%) with other sequences in GenBank with accession No**
RSm-3	Sonada	506	**LN823958**	99% with *Rhizobium etli* EBRI 21-**AY221176.**1
Rh K-1	Kurseong	487	**LN823959**	100% with *Rhizobium phaseoli* strain DAH2-2 –**KU 862348.1**
Rh NBU-8	Siliguri	489	**LN823960**	100% with *R leguminosarum* strain PB 162-**EF 525219.1**
Rh KPG-5N	Kalimpong	488	**LN823961**	100% with *R etli* strain CFN 42-**NR 029184.1**
Rh SAM-12	Samsing	485	**LN823962**	100% with *R etli* strain CFN 42- **NR 029184.1**
RSv-1	Siliguri	488	**LN823963**	100% with *R leguminosarum* strain PB 162-**EF 525219.1**
Rh MIR-6	Mirik	477	**LN823964**	100% with *R tropici* strain Hm 7-**KU 163458.1**
RMa-13	Malda	513	**LN 833880**	100% with *R leguminosarum* strain PB 162-**EF 525219.1**
Rh SKM	Sikkim	496	**LN 833881**	98% with *R leguminosarum* strain CCBAU 15396-**GU 552880.1**
Rh RGJ	Raiganj	472	**LN 833882**	98% with *R paranaense* strain PRF 54**,** complete sequence**- AF 260275.2**
Rh CBR	CoochBehar	512	**LN 833883**	99% with *Rhizobium* sp.Trp-3-**AF 511491.1**

Fig 4.27. Dendrogram generated by RAxML representing the relationship of the eleven *Rhizobium* strains with the standard GenBank partial sequences of rhizobial 16SrDNA . A high resolution figure of this tree has been given in supplementary figure SF1.

So far the strains of *Rhizobium* species are concerned, they were sharply divided into two clades. In one clade which is close to *Agrobacterium* there were two strains of *R. gallicum* while in the other clade all the other standard

Rhizobium strains clustered together including the strains isolated (LN833880-LN833883and LN823959-LN823964) (Fig 4.27). While in that particular clade there was one subclade with two strains of *R. giardinii*.

4.11. Whole genome sequencing and assembly

The *Rhizobium* strain RSm-3 was sent for the whole genome sequencing. The sequencing was done in collaboration with Prof. Louis S Tisa of Hubbard Genome Center, University of New Hampshire, Durham, NH. A Bio project ID (PRJNA 296467) was created in NCBI for the whole genome sequence of *Rhizobium* strains.

The BLASTN program revealed the whole genome sequence of RSm-3 to match with the complete genome sequence of *R. leguminosarum* bv *trifolii* WSM2304 with 96% identity and E-value 0.0.

During the whole genome sequencing of the strain RSm-3, a standard Illumina shotgun library was constructed and sequenced using the Illumina HiSeq2000 platform, which generated 1585078 reads and mean length of original fragment, reads in bases was 214.5. The final draft Assembly contained 58 contigs with an N50 of 313 kb. The number of contigs per Mb was found to be 8.4. Total size of the genome was estimated to be 7.3 Mbp. The GC content of the fragment read was found to be 60.6%. revealing the GC-richness of this particular strain. At K=25 scale, 11% of the genome was estimated to be repetitive.

The IMG annotation with the Genome ID 2684623037(Fig. 4.28) revealed the total protein coding genes to be 6564, RNA (53), tRNA (46), 5SrRNA (2). Only, one each of 16SrRNA and 23SrRNA small subunit ribosomal RNA was found. The details has been depicted in table 4.18 while the complete IMG annotation data has been provided in the supplementary table ST1. This annotation was quite similar with the result obtained from RAST Annotation Server. However, the bioinformatics study described later has been done on the basis of IMG result.

4.12. Bioinformatics study

4.12.1. *Genes involved in Nitrogen Fixing pathway*

4.12.1.1. *Synonymous codon usage pattern and heterogeneity*

The main intention of the study on the codon usage pattern was to estimate the level of heterogeneity in codon use.

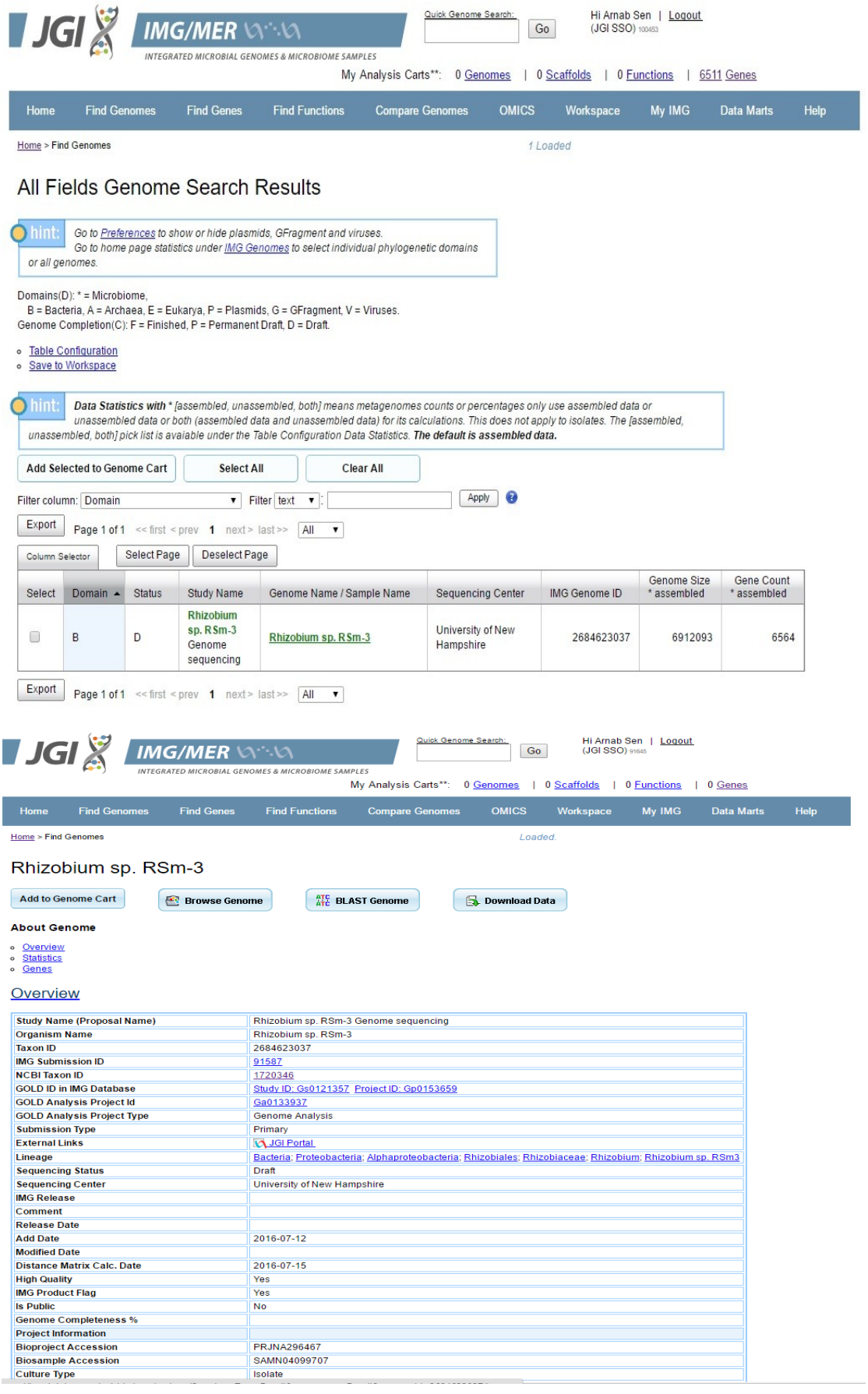

Fig 4.28. Snapshot of *Rhizobium* sp. RSm-3 available in IMG/ER database.

Table 4.18. Annotation and assembly report of *Rhizobium* sp. RSm-3 as per IMG.

Genome Id	**2684623037**
Total number of original fragment reads	1585078
Estimated genome size in bases	7305915
Number of contigs	58
Number of contigs per Mb	8.4
Number of scaffolds	60
Total contig length	6911122
Total no. of protein coding gene	6564
Total no. of RNA	53
tRNA	46
5s	2
16s	1
23s	1
Protein coding genes with function prediction	5371
Protein coding genes with enzymes	1563
Protein coding genes connected to Transporter Classification	1091
Protein coding genes connected to KEGG Orthology (KO)	3275
Protein coding genes connected to MetaCyc pathways	1376
Protein coding genes with COGs	4802

The genomes of four *Rhizobium* strains including the strain RSm-3 were analyzed for codon heterogeneity using parameters like GC content, GC3 content and Nc. The effective number of codons (Nc) versus GC3 plots has been suggested to be an important means of investigating codon usage variations among genes in the same genome (Peden, 2000). Fig.4.28 depicts the Nc/GC3 plots for studied genomes. In *Rhizobium* strains, the effective number of codon values ranged from 21 ± 2 to 61 ± 0 for all five genomes suggesting that these high GC-rich genomes exhibited considerable heterogeneity in codon usage. The position of genes associated with Nitrogen fixation (NFGs) in *Rhizobium* has been shown in the Nc/ GC3 plot. Most of the ribosomal protein genes were strongly clustered at lower right end of the plots indicating a significantly strong codon bias in these genes. The NFGs were also shown in the plots with red colored dots which have clustered more or less strongly with the ribosomal protein genes. All *Rhizobium* strains under consideration showed similar pattern of distribution of NFGs in Nc/GC3 plot. GC3 versus Nc plots can be effectively employed to investigate the factors underlying variations in codon usage patterns among genes and genomes. Wright (Wright, 1990) has suggested that, all the genes concerned would fall on the 'continuous Nc plot curve' or popularly the 'rainbow curve' if the

codon usage of a genome is completely governed by only GC compositional constraint. It was found that majority of the genes were well below the rainbow curve. This indicated that there might be other crucial factors

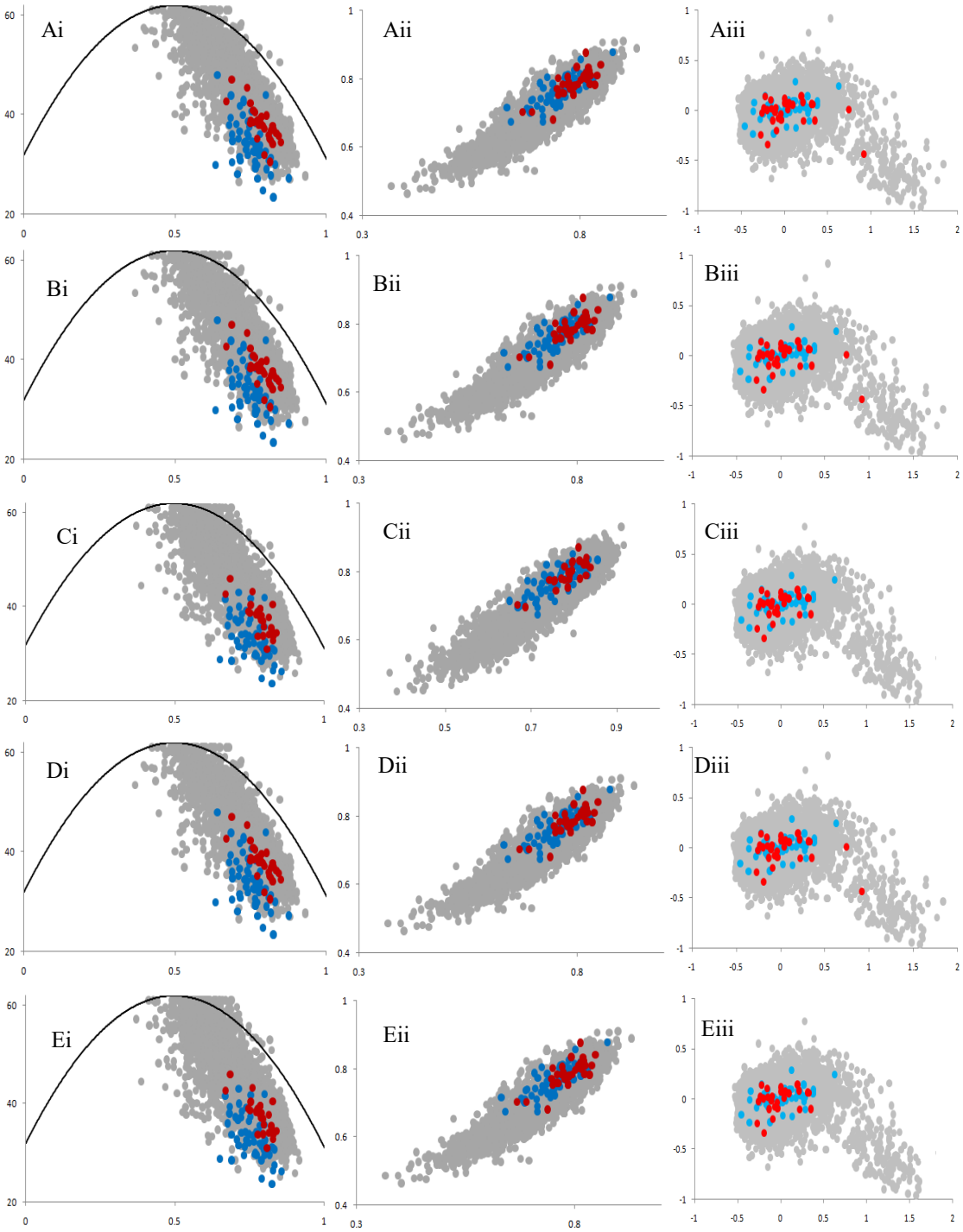

Fig 4.29. Codon usage analysis of Nitrogen fixing genes in 5 *Rhizobium* starins. First column represents the Nc vs GC3 plot where X axis is GC3 and Y axis is Nc value; second column represents the CAI vs GC3 plot where X axis is GC3 and Y axis is CAI and the third column represents the RSCU major axes values; A: *R. leguminosarum* bv. viciae 3841, B: *R. leguminosarum* bv. trifolii WSM1325 , C: R. etli bv. *phaseoli* IE4803, D: *R. etli* bv. *mimosae* Mim1, E: *Rhizobium* sp. RSm-3

apart from the influence of compositional constraint which is governing the codon usage patterns of these strains. It has been proposed by (Comeron and Aguade, 1998) that genes with low Nc values (<40) are under the influence of a strong codon bias and would cluster at the lower part of the plot. Here the ribosomal protein genes have clubbed at the extreme lower part of the plot whereas, NFGs are not present at the very lower end of the plot indicating that, compositional constraint is not the sole factor governing the codon usage tendency of these genes.

4.12.1.2. *Expression pattern analysis*

In CAI vs GC3 plots, nitrogen fixation related genes were clustered more or less with the ribosomal protein genes depicting their moderately high expression level (Fig. 4.28). The CAI values of the nitrogen fixing genes were correlated with Nc, Fop and GC3 values and correlation coefficient is provided in the Table 4.19A. A strong positive correlation between CAI and Fop was observed in all the cases revealing the utilization of the majority of optimal codons by NFGs. CAI values also showed a significant positive correlation with GC3 values. This is attributed to the fact that, a strong compositional constraint was working on NFG expression pattern.

4.12.1.3. *RSCU data analysis*

RSCU values obtained from Codon W were subjected to multivariate statistical analysis (correspondence analysis) for investigating the source of the complex codon usage property of *Rhizobium*. Scatter plots, on the basis of RSCU values of two major axes distinctly reflected the pattern of nitrogen fixing genes. Most of the selected genes representing the high CAI values grouped together with the ribosomal genes (Fig. 4.29). This tendency indicated towards the role of translational selection acting on the NFGs.

Axis 1 of RSCU was also found to correlate strongly with Fop, GC3 and CAI for nitrogen fixation related genes in all the strains. This correlation pointed towards the preferred usage of GC3 rich optimal codons in N_2 fixing pathway related genes which means a certain level of translational selection was also acting upon these genes.

Hence, we may hypothesize that, GC compositional constraints along with the translational selection have an influence on the codon usage as well as

Fig 4.19. Spearman's rho correlation of Rhizobium sp. RSm-3; A. Nitrogen fixing genes; B.Siderophore forming genes; C.IAA producing genes of *Rhizobium* sp. RSm-3;** indicates that correlation is significant at the level of 0.01 (2 tailed).

A. *Rhizobium* sp. RSm-3 Nitrogen fixing genes

	CAI	GC3	FOP	Nc
CAI	1	0.78**	0.76**	-0.36**
GC3		1	0.66**	-0.30**
FOP			1	-0.28**
Nc				1

B. *Rhizobium* sp. RSm-3 Siderophore forming genes

	CAI	GC3	FOP	Nc
CAI	1	0.72**	0.70**	-0.29**
GC3		1	0.69**	-0.35**
FOP			1	-0.30**
Nc				1

C. *Rhizobium* sp. RSm-3 IAA producing genes

	CAI	GC3	FOP	Nc
CAI	1	0.70**	0.73**	-0.28**
GC3		1	0.65**	-0.51**
FOP			1	-0.25**
Nc				1

expression pattern on Nitrogen fixing genes (NFGs) in *Rhizobium*.

4.12.2. *Genes involved in Siderophore biosynthetic pathway*

4.12.2.1. *Expression pattern analysis*

Codon adaptation index is one of the major preferred techniques for analyzing the codon usage bias of an organism. CAI actually measures the deviation of a gene from a reference set of gene. Here, we have used the ribosomal protein genes as our reference set for calculating the codon adaptation index (CAI) and have plotted the CAI values against the GC3 values.

From CAI vs GC3 plots we observed, the moderately high expression level of siderophore biosynthetic genes (SBGs) clustered relatively with the ribosomal protein genes (Fig.4.30). The CAI values of the genes of interest were correlated with Nc, Fop and GC3 values and correlation coefficient has been given in the Table 4.19B. A significant positive correlation between CAI, GC3 and Fop indicated towards the preference of GC rich optimal codons signifying a notable influence

of GC compositional constraint acting on the SBGs.

4.12.2.2. *Synonymous codon usage pattern and heterogeneity*

The codon usage pattern estimates the level of heterogeneity in codon usage.

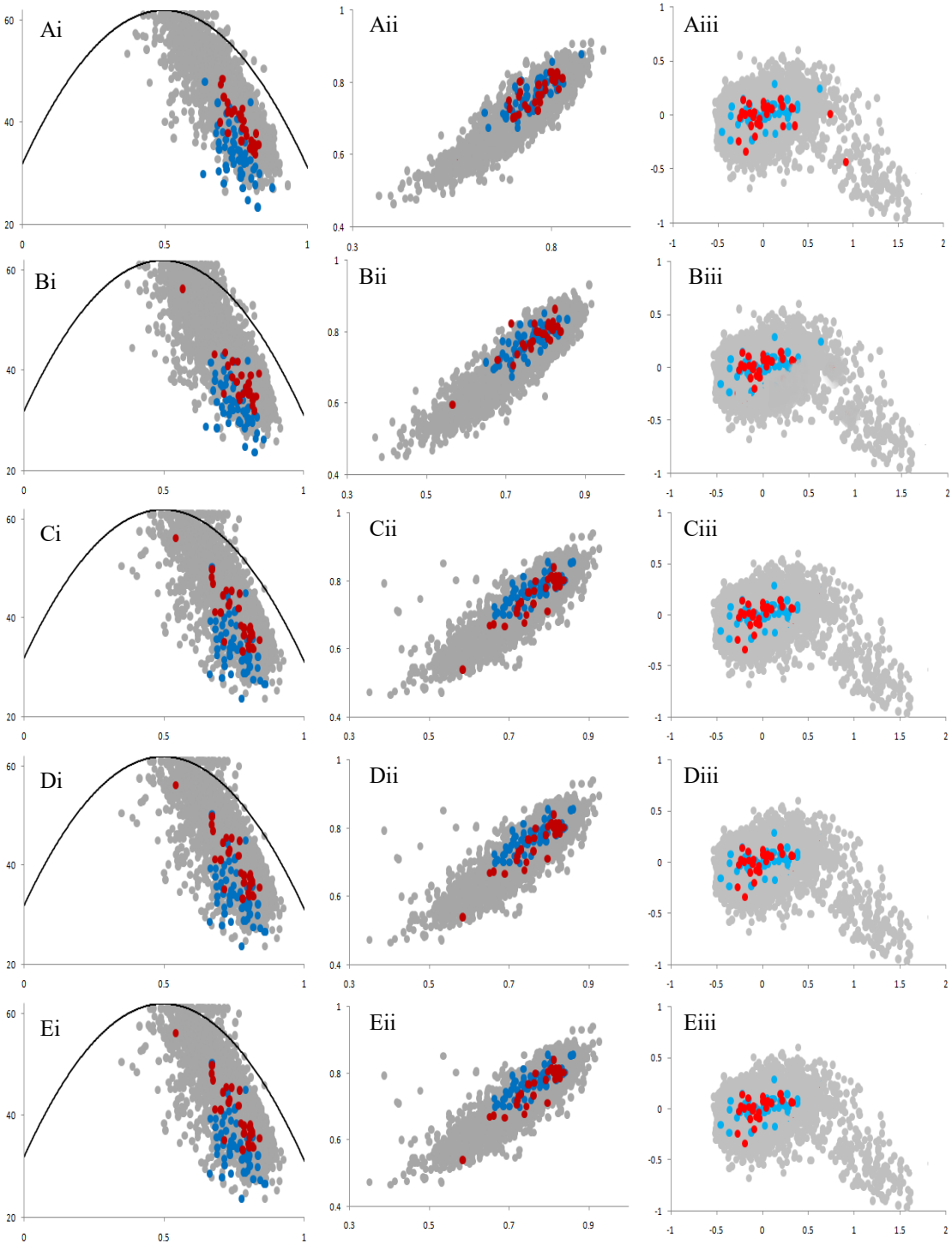

Fig 4.30. Codon usage analysis of Siderophore forming genes in 5 *Rhizobium* starins. First column represents the Nc vs GC3 plot where X axis is GC3 and Y axis is Nc value; second column represents the CAI vs GC3 plot where X axis is GC3 and Y axis is CAI and the third column represents the RSCU major axes values; A: *R. leguminosarum* bv. viciae 3841, B: *R. leguminosarum* bv. trifolii WSM1325 , C: R. *etli* bv. *phaseoli* IE4803, D: R. *etli* bv. *mimosae* Mim1, E: *Rhizobium* sp. RSm-3

The SBGs of five *Rhizobium* strains were analyzed using the parameters like GC content, GC3 content and Nc. The effective number of codons (Nc) versus GC3 plot is an important means of investigating codon usage variations among genes in the same genome (Peden, 2000). In *Rhizobium* strains, the effective number of codon values range from 21 ± 2 to 61 ± 0 for all five genomes suggesting that these highly GC-rich genomes exhibited considerable heterogeneity in codon usage Fig.4.30. The location of genes associated with siderophore formation in *Rhizobium* has been shown in the Nc/GC3 plot (Fig. 4.30). Most of the ribosomal protein genes clustered at lower end of the plots indicating a significantly strong codon bias in these genes. The genes associated with siderophore formation, were also shown in the plots with red colored circles which were not very much clustered with the ribosomal genes. Wright (1990) suggested that GC3 versus Nc plot can be effectively employed to investigate the factors underlying variations in codon usage patterns among genes and genomes. If the codon usage of a genome is solely governed by GC compositional constraint, then all the genes concerned would fall on the continuous Nc plot curve. Here, majority of the siderophore forming genes were found to lie well below the curve signifying that apart from the influence of compositional constraint, there might be other crucial factors that govern the codon usage pattern.

4.12.2.3. *RSCU data analysis*

The statistical Spearman's rank correlation analysis has pointed the Axis1 to be the major axis in governing the codon usage pattern. Axis 1 of the SBGs has showed a strong positive correlation with Fop, CAI and GC3. This strong positive correlation has pointed towards the pivotal role of translational selection of these targeted genes i.e. these genes with considerably high expression level favor GC3 rich optimal codons to improve their translational skill.

Thus, from the above study it may be concluded that both compositional constraint as well as translational selection are acting together upon the siderophore biosynthetic genes causing their relatively higher expression level.

4.12.3. *Genes involved in IAA production pathway*

4.12.3.1. *RSCU data analysis*

RSCU data for all the strains were

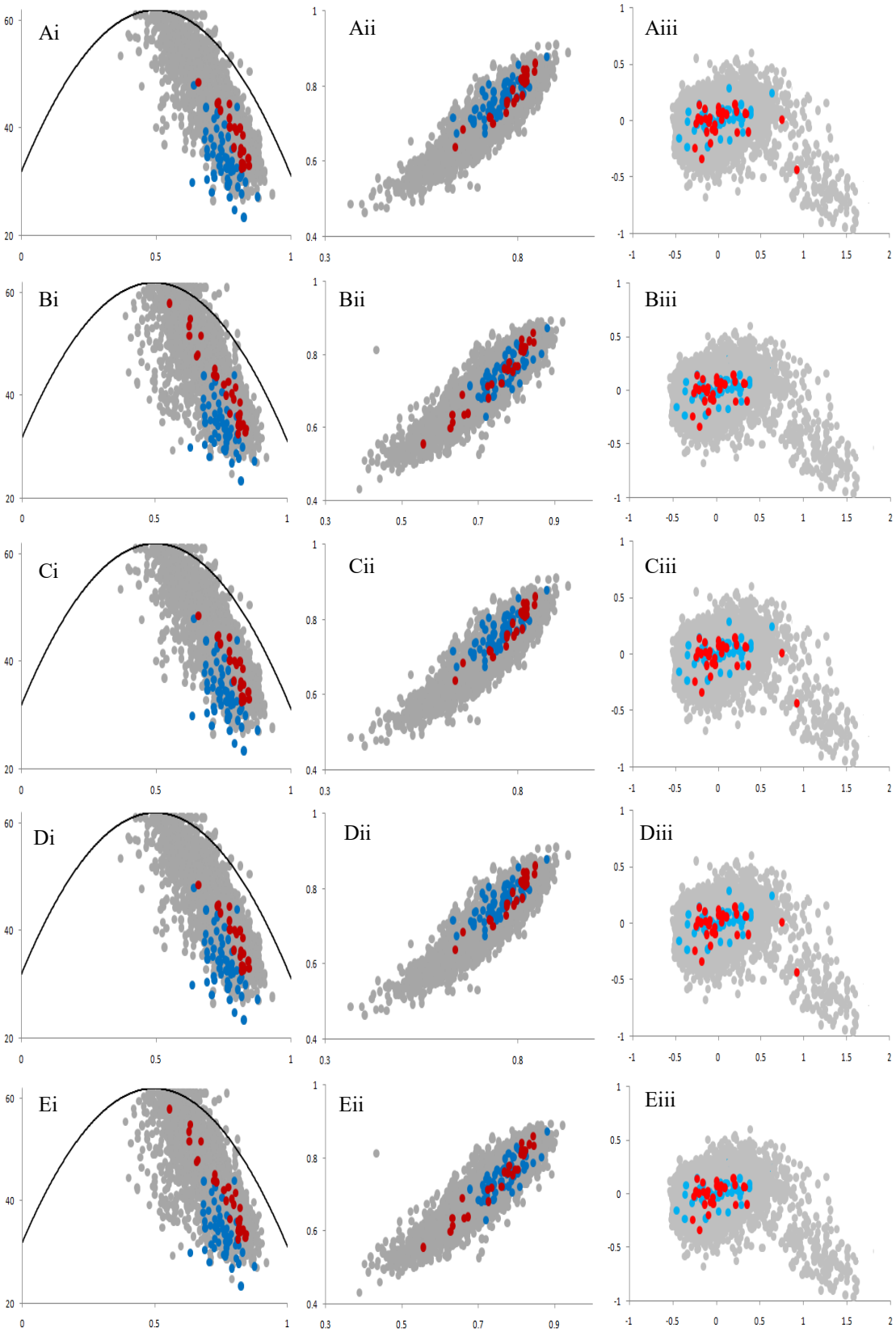

Fig 4.31. Codon usage analysis of IAA producing genes in 5 *Rhizobium* starins. First column represents the Nc vs GC3 plot where X axis is GC3 and Y axis is Nc value; second column represents the CAI vs GC3 plot where X axis is GC3 and Y axis is CAI and the third column represents the RSCU major axes values; A: *R. leguminosarum* bv. *viciae* 3841, B: *R. leguminosarum* bv. *trifolii* WSM1325 , C: *R. etli* bv. *phaseoli* IE4803, D: *R. etli* bv. *mimosae* Mim1, E: *Rhizobium* sp. RSm-3

subjected to multivariate statistical analysis (correspondence analysis) with a motive to address the sources of such complex codon usage tendencies. Scatter plots on the basis of RSCU values for *Rhizobium* distinctly reflected the pattern of IAA producing genes (IPGs). Most of the selected genes representing the higher CAI values flocked together with the ribosomal genes (Fig.4.31). Such tendencies of clustering pointed towards a mighty role of translational selection to be acting on the genes with high expressivity.

Axis 1 of RSCU was also found to correlate strongly with Fop for IAA production related genes in all the strains. Such high correlation hinted towards the variation of the genes based on their preference in usage of optimal codons. It could also be concluded from the substantial positive correlations of CAI with Fop in all the strains that the genes with high levels of expression preferentially used a particular subset of optimal codons to enhance the translational knack in IPGs.

4.12.3.2. *Synonymous codon usage pattern and heterogeneity*

The main motive of this study on the codon usage pattern was to estimate the level of heterogeneity in codon use. The genomes of four *Rhizobium* strains were analyzed for codon heterogeneity using the parameters like GC content, GC3 content and Nc. The effective number of codons (Nc) versus GC3 plots has been suggested to be an important means of investigating codon usage variations among genes in the same genome (Peden, 2000). Figure 4.31 depicts the Nc/GC3 plots for studied genomes. In *Rhizobium* strains, the effective number of codon values range from 21 ± 2 to 61 ± 0 for all four genomes suggesting that these highly GC-rich genomes exhibited considerable heterogeneity in codon usage. The location of IPGs in *Rhizobium* has been shown in the Nc/ GC3 plot. Most of the ribosomal protein genes strongly clustered at lower end of the plots indicating a significantly strong codon bias in these genes. The IPGs were also shown in the plots with red colored circles which have clustered more or less strongly with the ribosomal protein genes. All *Rhizobium* strains under consideration showed similar pattern of distribution of selected genes in Nc/GC3 plot.

GC3 versus Nc plots can be effectively employed to investigate the factors

underlying variations in codon usage patterns among genes and genomes. According to Wright(1990), if the codon usage of a genome is solely governed by GC compositional constraint, then all the genes concerned would fall on the continuous Nc plot curve. Majority of the genes were found to lie well below the curve signifying that apart from the influence of compositional constraint, there might be other crucial factors that govern the codon usage pattern (Fig.4.31). A clear trend was also evident from the plot that the ribosomal proteins, expected to show high levels of expression during cell division and growth, clustered at the extreme bottom of the plot. It has been proposed by Comeron and Aguade (1998) that genes showing low values of Nc (<40) are under the influence of a strong codon bias, that can merely be explained as an outcome of pure compositional constraint.

4.12.3.3. *Expression pattern analysis*

In CAI vs GC3 plots, IPGs were clustered more or less with the ribosomal protein genes depicting their moderately high expression level (Fig.4.31). The CAI values of these genes were correlated with Nc, Fop and GC3 values and correlation coefficient is provided in the Table 4.19C. A significant positive correlation between CAI and Fop revealed that, IPGs are utilizing the majority of the optimal codons. CAI value also showed a significant positive correlation with GC3 values thus, emphasizing the impact of

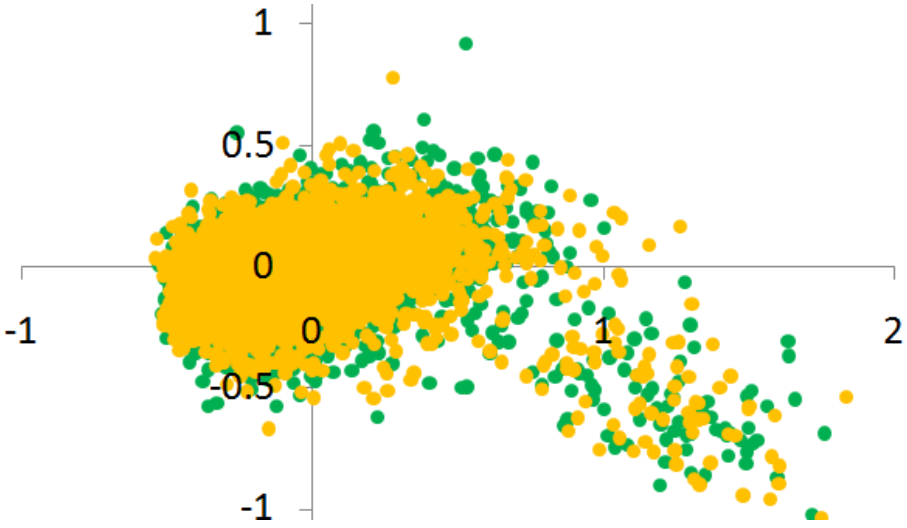

Fig 4.32. Absence of replication-transcriptional selection in *Rhizobium* sp. RSm-3. Yellow dots representing the genes present on leading strand and green dots represents the genes on lagging strand

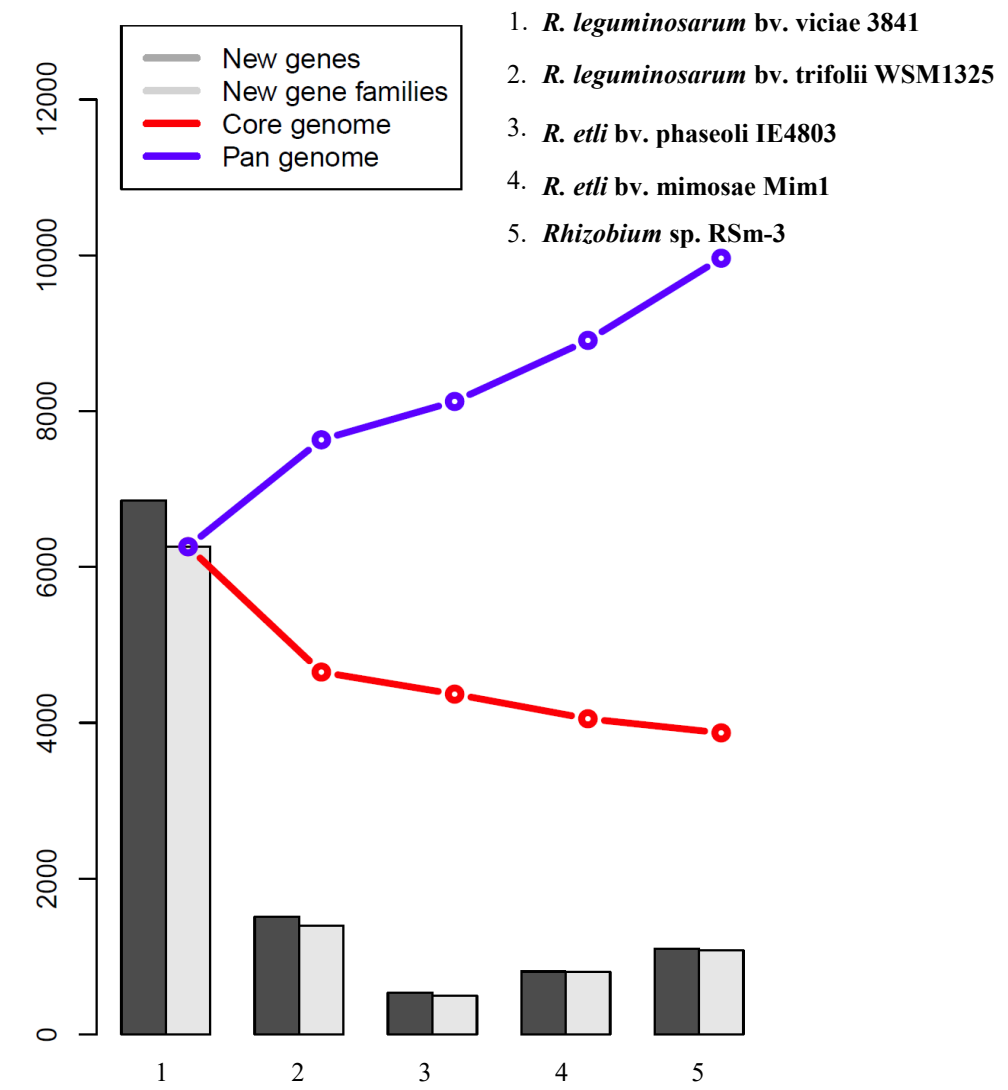

Fig 4.33. Pan-core plot of 5 *Rhizobium* strains. The blue lines indicate the pan genome count and red lines indicate the core gene count

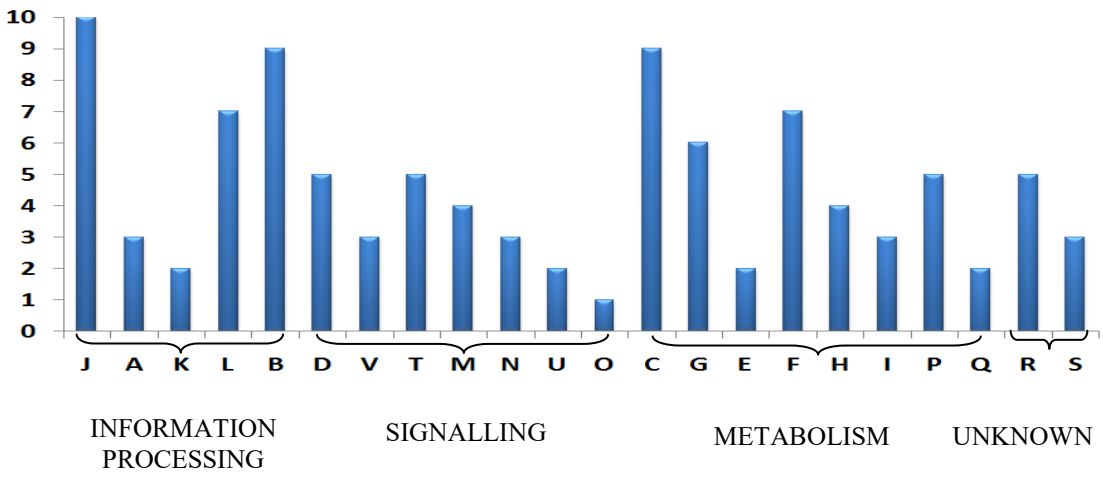

Fig 4.34. COG analysis of *Rhizobium sp. RSm*strains.

compositional constraint on the expression pattern of genes involved in IAA production.

Hence, we may hypothesize that, GC compositional constraints along with the translational selection have an influence on the codon usage as well as expression pattern on IPGs in *Rhizobium*.

4.12.4. *Comparative Genomics*

4.12.4.1 *Replicational transcriptional (RT) selection*

To investigate whether 'replicational and transcriptional' selection pressure prevails on these genomes, the number of genes present in leading and lagging strand was calculated . No significant differences were found in the dataset indicating the absence of replicational selection. Moreover, highly expressed genes did not posses any leading strand bias, hence we may say that, transcriptional selection is also absent in the targeted genomes. To get a pictorial view of this fact, the RSCU values of leading and lagging strand were plotted where, they clubbed together without any distinct clade. Thus, it became evident that, studied *Rhizobium* genomes are not under replicational-transcriptional selection pressure (Fig. 4.32).

4.12.4.2.Blast Matrix

The blast matrix showed 4.1% duplication in *Rhizobium* sp. RSm-3 genomes. This newly sequenced genome showed maximum similarity with *Rhizobium leguminosarum* bv. *trifolii* WSM1325.

4.12.4.3 Pan and Core genome analysis

The pan core plot has been given in (Fig.4.33). A total of 9962 genes were accumulated in pan genome. However, the core gene count was reduced to 3871. The addition of more *Rhizobium* genomes will affect the pan- and core-gene number. The core genes were found to be the house keeping genes involved in some major metabolic and signal transduction pathways. The COG categories of these genes were under information storage and processing as well as cellular process and cell signaling group (Fig. 4.34).