Chapter 4
RESULTS & DISCUSSION

4.1. Isolation of The Bacterium

The cortical layers of actinorhizal root nodules are heavily contaminated with soil microorganisms. The growth rate of which is generally faster than the *Frankia*. So, the prerequisite for successful isolation of *Frankia* was to eliminate these contaminants without damaging the endophyte in the cortical tissue. Of the three procedure tried to isolate the endophyte, procedure III (Bose and Sen, 2006) was found as the most suitable for easy isolation of *Frankia.* In this procedure a series of mild and easily available surface sterilizing agents were used to eliminate these contaminants, which was followed by incubation in rich Qmod (Lalonde and Calvert, 1979) medium for 48 hr at 30°C temperature with 3% activated charcoal. The surface sterilizing agents used were useful against the bacterial and fungal contaminant and the vegetative bacterial and fungal cells could be killed at high rate but the chemical resistant bacterial spores might withstand this treatment and they might germinate later on in culture medium. When the nodule lobes were incubated with rich Qmod (Lalonde and Calvert, 1979) medium for 48 hr at 30°C temperature the spores might germinate and the nascent vegetative cells might be killed by subsequent chemical treatment (Bose and Sen, 2006).

Another problem in *Frankia* isolation was the presence of plant phenolics which leached out in the medium from the piece of nodule during isolation. The plant phenolics were potential inhibitor of the growth of *Frankia.* The activated charcoal minimized the effect of phenolics by absorbing it and thus increased the efficiency of *Frankia* isolation.

The advantage of using Qmod (Lalonde and Calvert, 1979) in the post isolation maintenance of the bacterium was its nutritional richness, in which any contamination can not remain in dormant condition. Thus, Qmod (Lalonde and Calvert, 1979) medium visibly ensure an absolute contamination free condition for pure culture. Define propionate minimal medium (DPM) (Baker and O'keefe,1984) is also a very good medium for maintenance of *Frankia* cultures but this medium have some disadvantages in isolation of the endophytes. The absence of nitrogen in the DPM (Baker and O'keefe,1984) helped selectively in the growth of *Frankia* while the spore forming contaminants remain in the dormant condition for a long period. These contaminants might germinate latter while transferring in nutritionally rich medium.

Frankia colonies came as minute cottony white dots at the bottom of the tube after one month incubation in dark. Each colony was collected and subcultured in separate tubes containing Qmod (Lalonde and Calvert, 1979) medium. Although several concentrations of $VitB_{12}$ and $VitB_1$ were played in isolation medium but Vit B_{12} at 0.2% concentration in aqueous solution (1mL/L) and Vit B_1 at 0.5% concentration in aqueous solution (1mL/L) gave the best result.

4.2 Germination of Seeds of Actinorhizal Plants

The seeds of *Alnus nepalensis* and *Casuarina equisetifolia* need different conditions for germination. No special treatment was needed for germination of *C. equisetifolia* seeds and they were germinated on sterile distilled water in floating condition.

Generally, the seeds of *A. nepalensis* need a special kind of treatment in the form of overnight soaking in aerated water for better germination. The treated and non-treated conditions, media nutrients and contamination of fungus do not have any effect on the duration of seed germination. In all the cases the seeds germinated within 4 weeks. Contaminations were reported in mineral rich medium under treated and non-treated conditions. The percentage of germination varied with the media used (Fig: 4.1) in *A. nepalensis* seeds. Maximum germination was observed in Hoagland media for both treated and non treated seeds while minimum in ½ Murashige and Skooge medium (MS) for treated and ½ woody plant medium (WPM)

for non treated seeds. The germination was probably inhibited by high nutrient concentrations. For treated seeds, maximum (80%) survival rate was reported in Hoagland media while minimum (0%) survival rate was reported in WPM. For non-treated seeds, maximum (100%) survival rate was reported in Hoagland while minimum (20%) survival rate was reported in half strength WPM. Moderate germination and survival rate with low contamination were reported in water used as germination medium (Fig 4.2).

The hormones had profound but variable effect on the seed germination and rooting of *A. nepalensis*. IBA and NAA+IBA were found to be more effective than NAA in terms of germination percent and rooting. Moderate germination percentages (above 50%) were observed in the media supplemented with IBA 1 and 2 mg/L and NAA+IBA 1+1 and 2+2 mg/L. Maximum (90 %) germination was observed with the application of IBA (1 mg/L) and NAA + IBA (1+1 mg/L). The germination percent increased with the increase of NAA concentration while declined with the increase of IBA and NAA + IBA concentrations. Good rooting and good growth of roots were observed in the media supplemented with IBA 3, 4 and 5mg/L and NAA + IBA (1+1 mg/L). The best result was recorded in the medium supplemented with 4mg/L IBA. In the medium supplemented with NAA + IBA (2+2 mg/L) rooting was poor but the roots showed good growth.

In the study of seed germination in sterilized vermicompost, good germination rate was reported within 10 days but the seedlings did not survive. This death event was due to poor root development. In double layer culture experiment both the germination percentage and survival rate were very low although the media contained required hormones as described in earlier experiments.

These experiments reveals that germination of *A. nepalensis* seedlings have specific hormonal requirements. These hormonal requirements are fulfilled by mycorrhizal or associative fungi during germination and growth in nature. In vermicompost the *Alnus* seedlings did not survive though there were plenty of nutrients. The nutrients were not absorbed due to poor root development which require external hormonal supply. This plant *A. nepalensis*, thus begins its life with an association with fungi and at its

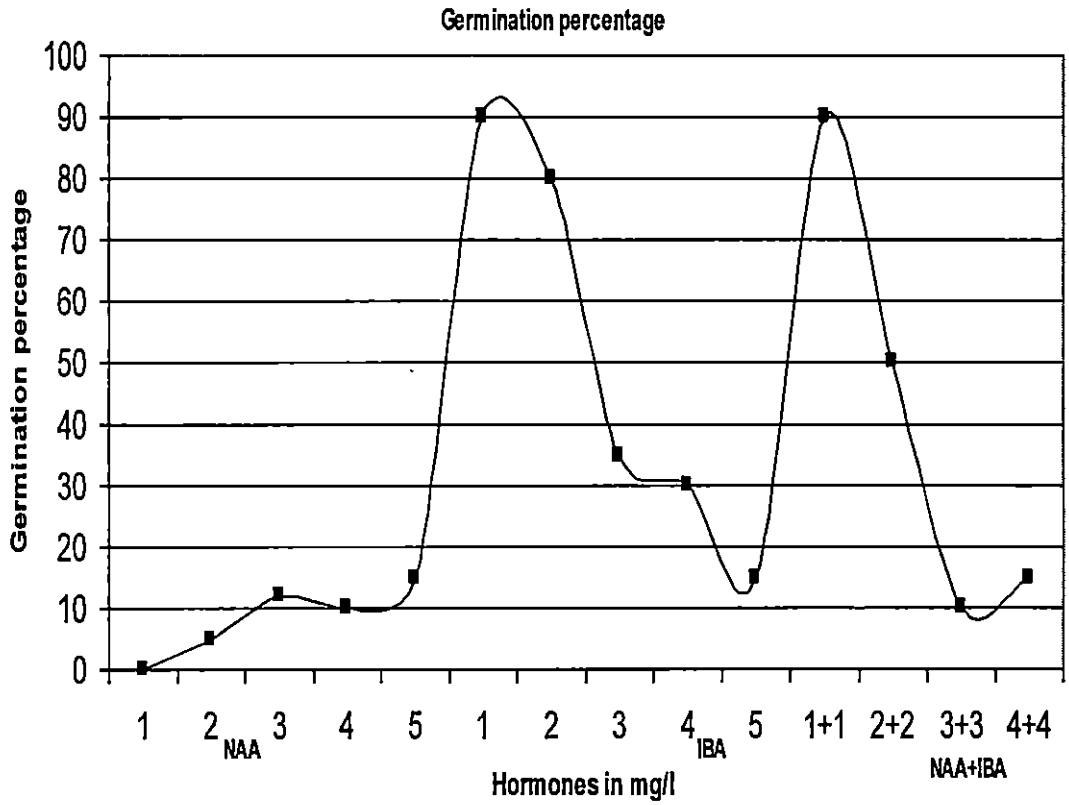

Fig 4.1: Effect of various hormone concentrations on *A. nepalensis* seed germination percentage.

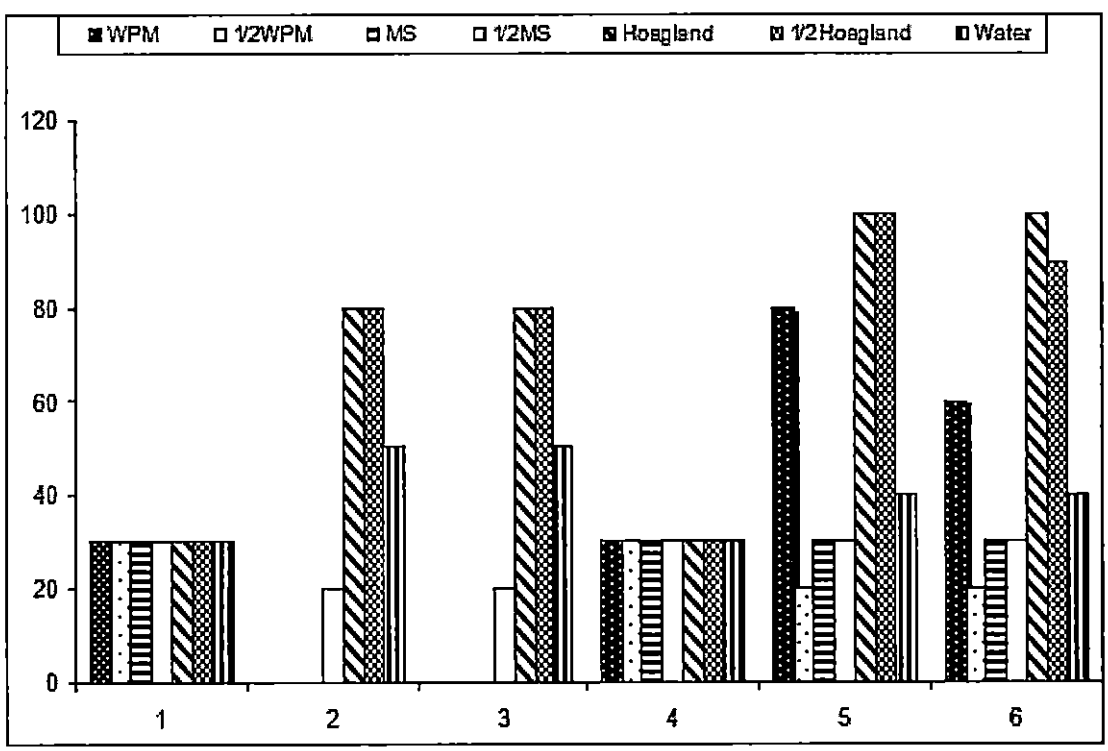

Fig 4.2: Generation time (G time), Generation percentage and survival rate of treated and untreated *A. nepalensis* seeds in different medium.

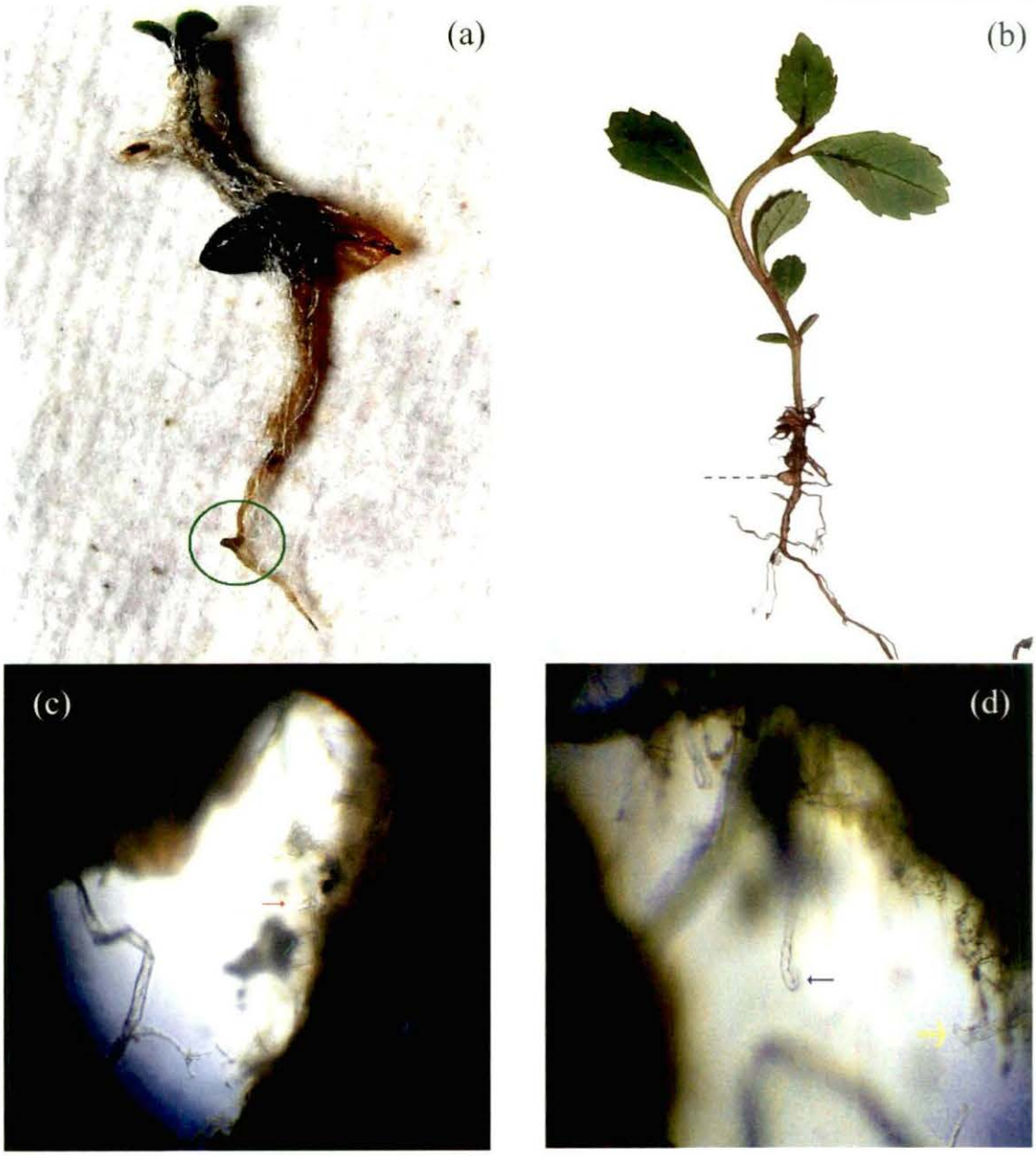

Fig 4.3: a) A young *C. equisetifolia* seedling with nodule; b) A young *A. nepalensis* seedling with nodule; c) A young root hairs show ignition of root hair deformation due to *Frankia* infection: d) A young root hairs show typical hook shaped root hair deformation due to *Frankia* infection.

maturity make another association for survival with an actinomycetes bacteria *Frankia*. *C. equisetifolia,* did not have this problem.

4.3 Plant Infectivity Test

The characteristic root hair deformation and visible nodule formation (Fig 4.3) were observed both in *A. nepalensis* and *C. equisetifolia* seedlings grow in aseptic condition. This indicates the presence of *Frankia* in the medium.

4.4 Colony Morphology

Medium used: Defined propionate minimal medium (Baker and O'keefe, 1984)

Reaction pH: 7

A. Size: Filamentous.

Temperature: 28^{0}C. **Age:** 30 days

B. Motility : Not observed

C. Sporangia and spores: Present, sporangia multilocular, spore round shaped smooth walled in older culture. 2mM Pb increase sporulation.

D. Agar colonies :

Temperature : 28^{0}C

Age : 14 days.

Location : Deep colony

Form : Spindle shaped

Edge : Filamentous

Optical character: Opaque

Pigmentation : Red in 2mM Pb concentration.

E. Broth:

Temperature : 28^{0}C

Age : 14 days.

Odor : None

Clouding : Absent

Surface growth: Absent

Sediments : Flanky

Amount of sedimentation: Scanty

F. Forms and arrangement: Filamentous

G. Gram staining : Positive

H. Biological relationship: Symbiotic to actinorhizal plants.

I. Isolated From : DPM (Baker and O'keefe, 1984) lead plates containing 2mM Lead. (Bose and Sen, 2005)

4.5 Field Emission Scanning Electron Microscopy

The hyphae, sporangium, and vesicles are the most important structures of *Frankia*. The multilocular sporangium, round shaped vesicle structures were found. The structure of sporangium was ±1.5 x 0.7 µ. The mean diameter of the hyphae was ± 0.5µ. The mean diameter of vesicle was ± 0.7µ. These results were nicely fitted with a classical frankial structure (Fig 4.4a & 4.4b).

4.6 Biochemical Characters of *Frankia*

Eleven *Frankia* strains were selected and used in the study of physiology of the bacterium. Out of these eleven strains six were isolated from *C. equisetifolia* and the rest from *A. nepalensis.*

The three *Frankia* strains (CeSi10, CeSi11, CeSi12) isolated from *C. equisetifolia* showed rapid decarboxylation activity with propionate and pyruvate and very slow decarboxylation activity with acetate and citrate. Decarboxylation leads to a rise in pH assessed as change in colour of the indicator from yellow - orange to red. These strains showed high protease activity which were demonstrated by the liquefaction of gelatin. None of these strains showed β

-glucosidase activity. They could utilize a variety of organic acids and a few carbohydrates. They had utilized propionate, acetate, succinate, sucrose and fructose as sole carbon source as they produce visible colonies within 60 days of incubation, but they can not utilize lactose, glucose, pyruvate, mannitol, tween80 and citrate.

The other three *Frankia* strains (CeSt2, CeSt5, CeSt9) isolated from *C. equisetifolia* showed no decarboxylation activity with propionate, pyruvate, acetate and citrate. None of these strains showed β-glucosidase activity, though they showed very slow protease activity. They could utilize propionate, acetate and succinate as sole source of carbon but they could not utilize glucose, pyruvate, mannitol, tween 80, citrate, sucrose, fructose and lactose. All the above experiments were done in triplicate and same results were found . The results are summarized in table 4.1 and table 4.2.

It was very interesting to note that these *Frankia* strains could not utilize the tween compounds which is a very common carbon source for *Frankia.* But non utilization of tween compound is not uncommon in *Frankia* strains. Several well recognized strains could not recognize tween compounds which include $ACN1^{AG}$, EAN1pec, and EuIIc etc. A possible explanation of the difference between the two groups of *Frankia* is that they lack, an active carbohydrate transport system (Prof. Normand, personal communication).

From the above result it could be concluded that the three strains namely CeSi10, CeSi11 and CeSi12 belongs to the physiological group 'A' of the *Frankia* which is more saprophytic than symbiotic while the other strains namely CeSt2, CeSt5, CeSt9 belongs to the physiological group 'B' of the *Frankia* which are physiologically less active and more symbiotic. Overall the strains utilize the propionate and acetate as best carbon source. So in this area results showed diversity among the *Frankia* on the basis of standard physiological parameters. These tests can be a useful addition to the methods used in differentiating *Frankia* strains isolated from *C. equisetifolia.*

Similar physiological experiments were also performed with the *Frankia* strains isolated from *A. nepalensis.* Two *Frankia* strains (AnMr1, AnMr 5) isolated from the root nodules of *A. nepalensis* growing in Darjeeling Hills showed rapid decarboxylation activity with propionate and pyruvate and

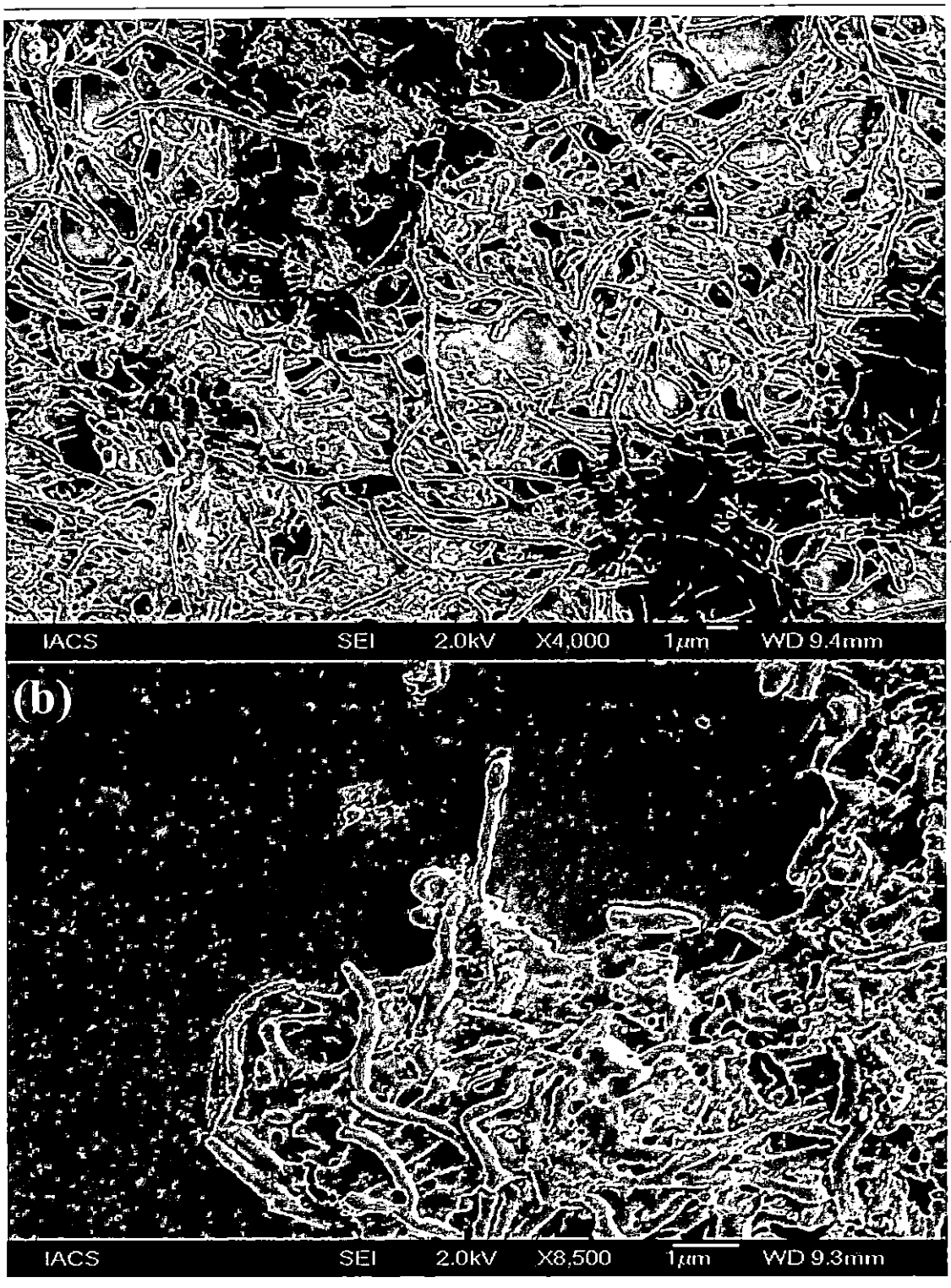

Fig 4.4: a) FESEM picture of multilocular sporangia of *Frankia;* b) FESEM picture of vesicle of *Frankia*

another three *Frankia* strains (AnMr2, AnMr2a AnMr2b) isolated from the root nodules of the same plants also growing in the same area showed no decarboxylation activity with propionate and pyruvate and the strains AnMr2b showed slow decarboxylation activity with acetate and citrate as

Table 4.1: Utilization of different carbon source by *Frankia* isolated from *A. nepalensis* and *C. equisetifolia.*

	Alnus specific strains				
	AnMr1	AnMr5	AnMr2	AnMr2b	AnMr2a
Propionate	+	+	+	+	+
Pyruvate	+	+	+	+	+
Acetate	+	+	+	+	+
Succinate	+	+	+	+	+
Glucose	(-)	(-)	(-)	(-)	(-)
Sucrose	(-)	(-)	(-)	(-)	(-)
Lactose	(-)	(-)	(-)	(-)	(-)
Fructose	(-)	(-)	(-)	(-)	(-)
Tween 80	+	+	+	+	+
Glucose + Tween 80	(-)	(-)	(-)	(-)	(-)
Mannitol	+	+	+	+	+
Citrate	+	+	+	+	+

	Casuarina specific *Frankia* strains					
	CeSi 10	CeSi 11	CeSi 12	CeSt 2	CeSt 5	CeSt 9
Propionate	+	+	+	+	+	+
Pyruvate	(-)	(-)	(-)	(-)	(-)	(-)
Acetate	+	+	+	+	+	+
Succinate	+	+	+	+	+	+
Glucose	(-)	(-)	(-)	(-)	(-)	(-)
Sucrose	+	+	+	(-)	(-)	(-)
Lactose	(-)	(-)	(-)	(-)	(-)	(-)
Fructose	+	+	+	(-)	(-)	(-)
Tween 80	(-)	(-)	(-)	(-)	(-)	(-)
Glucose + Tween 80	(-)	(-)	(-)	(-)	(-)	(-)
Mannitol	(-)	(-)	(-)	(-)	(-)	(-)
Citrate	(-)	(-)	(-)	(-)	(-)	(-)

+ = Visible colony occur within 30 days of incubation; (-) = No visible colony occur within 30 days of incubation;

shown earlier by *Frankia* isolated from *C. equisetifolia.* The AnMr1 and AnMr5 show high protease activity which is demonstrated by the liquefaction of gelatin while rest of the strains shows very slow protease activity. None of these strains showed β-glucosidase activity. These strains could utilize a variety of organic acids and a few carbohydrates. They utilized propionate, acetate, succinate, pyruvate, mannitol, tween80 and citrate as sole carbon source as they produce visible colonies within 60 days of

Table 4.2. Physiological characteristics of *Frankia* isolated from *A. nepalensis* and *C. equisetifolia*

	ACN1[AG*]	AnMr1	AnMr5	AnMr2b	AnMr2	AnMr2a	CeSi10	CeSi11	CeSi12	CeSt2	CeSt5	CeSt9
Dec. of acetate	(-)	+	+	+	+	+	+	+	+	(-)	(-)	(-)
Dec. of propionate	(-)	++	++	(-)	(-)	(-)	++	++	++	(-)	(-)	(-)
Dec. of citrate	(-)	+	+	+	+	+	+	+	+	(-)	(-)	(-)
Dec. of pyruvate	(-)	++	++	(-)	(-)	(-)	++	++	++	(-)	(-)	(-)
Protease activity	++	++	++	(-)	(-)	(-)	++	++	++	(+)	(+)	(+)
β-glucosidase activity	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)

Dec. = Decarboxylation; + = Positive result (with in two to three weeks); ++ = High activity (positive result with in two weeks); (+) = Very slow (positive result with in four weeks); (-) = Negative result with in 42 days of incubation; *= Reference strain.

incubation, but they could not utilize lactose, glucose, fructose and sucrose. All the above experiments were done in triplicate and same results were found. The results are summarized in table 4.1 and table 4.2.

From the above result it could be concluded that the strains isolated from the Darjeeling Hills belong to the physiological group 'A' of the *Frankia* which were more saprophytic than symbiotic. Overall the strains utilize the propionate and acetate as best carbon source. So, in this area we were able to detect the diversity among the *Frankia* on the basis of standard physiological parameters. These tests could be a useful addition to the methods used in differentiating *Frankia* strains isolated from *C. equisetifolia* and *A. nepalensis*.

4.7 Statistical Analysis of The Biochemical Data by Software POPGENE

The result of these physiological tests were analyzed by POPGENE and the diversity of *Frankia* were determined by Nei genetic diversity (Nei, 1987), Shannon index (Shannon, 1948; Weaver and Shannon, 1949), single locus component and two locus component (Brown and Feldman,

1981) and Wahlund effect (Brown and Feldman, 1981). Among the *Frankia* population the total genetic diversity had been found to be 0.281 (SD ± 0.0547), which showed low level of genetic diversity. The estimated gene flow within the population was zero. The coefficient of gene differentiation among population indicated what percentage of total genetic diversity came from interpopulation and in this case, there was no lateral gene transfer in *Frankia* strains. So, the entire genetic diversity came from intrapopulation source and hence the value of coefficient of gene differentiation was 1.

Shannon index is used to study the diversity of the population. It is assumed that all species are represented in the sample and they are randomly sampled. The advantage of this index is that it takes into the account the number of species and the evenness of the species. The index is increased either by having additional unique species or by having greater species evenness. The value of Shannon index will be maximum if the evenness present in the population is maximum. It has specific importance in the expression of equal proportion character in the sample rather than the total number of individuals. The Shannon index has moderate sensitivity towards the sample size. In the present study the value of Shannon index had been found as 0.3982 (SD= ± 0.3293), which indicated a moderate level of diversity.

Another important diversity measurement index used in this study was the single locus component and and two locus component. The single locus components are the average and the variation among the population in gene diversity and variance among the population in allel frequency. The two locus component include the mean and variance of disequilibria, the covariance of allel frequencies, over population and various interactions. Wahlund effect is used to explain the single locus component and and two locus component. It refers to the reduction in the heterozygocity in the population caused by subpopulation structure. The *Frankia* population showed Wahlund effect 2.7071 for single locus effect and 8.901 for the two locus effect. If the subpopulations have the same gene frequency the value of Wahlund effect will be zero. In this case the value represented very low level of genetic diversity among *Frankia* population.

The dendrogram Based on Nei's (1987) Genetic distance (Method = UPGMA Modified from NEIGHBOR procedure of PHYLIP Version 3.5) (Fig 4.5) showed that the *Frankia* population was divided in to two major clades. The first clade included the bacterium isolated from *C. equisetifolia* and the second clade included bacterium isolated from *A. nepalensis*. Each clade was again sub divided into two minor clades. This pattern of the dendrogram supported the presence of two physiological groups among the bacteria isolated from each host.

Thus from the result of Nei genetic diversity (Nei, 1987), Shannon index (Shannon, 1948; Weaver and Shannon, 1949), single locus component and two locus component (Brown and Feldman, 1981) and Wahlund effect (Brown and Feldman, 1981), it can be concluded that that the *Frankia* population present in this area shows very low genetic diversity and the total genetic diversity was intrapopulation in origin and divided into two different physiological group (Fig4.5). (Bose *et al.*, 2011)

4.8 Heavy Metal Salt Resistance Pattern

Heavy metal salt resistant colonies of *Frankia* appeared after around 14 days of incubation. The numbers of colonies were plotted against the increasing concentration of various heavy metal salts (Fig 4.6; 4.7) and the minimum inhibition concentrations (MIC) calculated (Table 4.3). Figure 4.6 shows the growth pattern of *Frankia* strains isolated from *C. equisetifolia* in presence of different metal salts. The results show that the strain was highly resistant to cadmium chloride (5mM), moderately resistant to cobalt chloride and lead nitrate (3mM) and less resistant to nickel chloride and copper sulphate (0.5 mM). This results were compared with the American strains and found that the present strain was much more tolerant to cadmium chloride and cobalt chloride. While the MIC value of USA strains CcI3 and Cc117 for $CdCl_2$ was 0.1 mM and for $CoCl_2$ 0.1 and 0.05 mM respectively. This strain showed a MIC value of 5 and 3 mM for $CdCl_2$ and $CoCl_2$ respectively. However, USA strains grew at an elevated concentration of $CuSO_4$, $NiCl_2$ and Pb $(NO_3)_2$ than the studied strains (Richard *et al.*, 2002). The heavy metal resistant *Frankia* colonies were re-isolated from various plates and preserved for further studies. Each experiments were repeated twice and were found to produce similar results. The strain used in the study

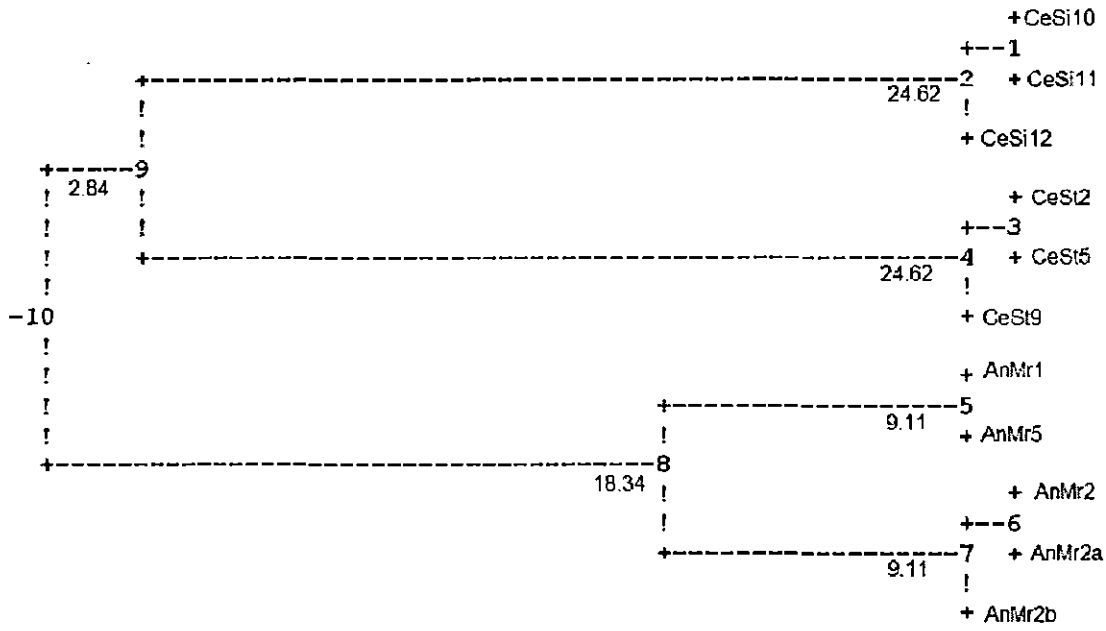

Fig 4.5: Dendrogram Based on Nei's (1987) Genetic distance showing the diversity of *Frankia* based on physiological results: Method = UPGMA Modified from NEIGHBOR procedure of PHYLIP Version 3.5. Numbers indicated in the bottom of the lines are root distance.

were found compatible with elevated concentration of cadmium, cobalt and lead salts.

Similarly, the same protocol was used for the heavy metal resistance pattern study in *Frankia* isolated from the *A. nepalensis*. Heavy metal salt resistant colonies of *Frankia* isolated from the *A. nepalensis* appeared similarly after around 14 days of incubation. The numbers of colonies were counted against the increasing concentration of various heavy metal salts and plotted in the graph. Fig 4.7 shows the growth pattern of *Frankia* strain AnMr1 in presence of different metal salts. The results show that the strains were highly resistant to cadmium chloride (2mM) and lead nitrate (5mM) moderately resistant to cobalt chloride (0.75mM) and less resistant to nickel chloride(0.05mM) and copper sulphate (0.5 mM). This results were also compared with the American strains (Richards *et al.*, 2002) and found that the present strains were much more tolerant to cadmium chloride and copper sulphate. While the MIC value of USA strains for $CdCl_2$ was 0.5 mM and for $CoCl_2$ 0.25, the strain, studied strains showed a MIC value of 2 and 0.5 mM for $CdCl_2$ and $CoCl_2$ respectively. However, USA strains grew at an elevated concentration of $NiCl_2$ and $Pb(NO_3)_2$ than the studied strain. The heavy metal resistant

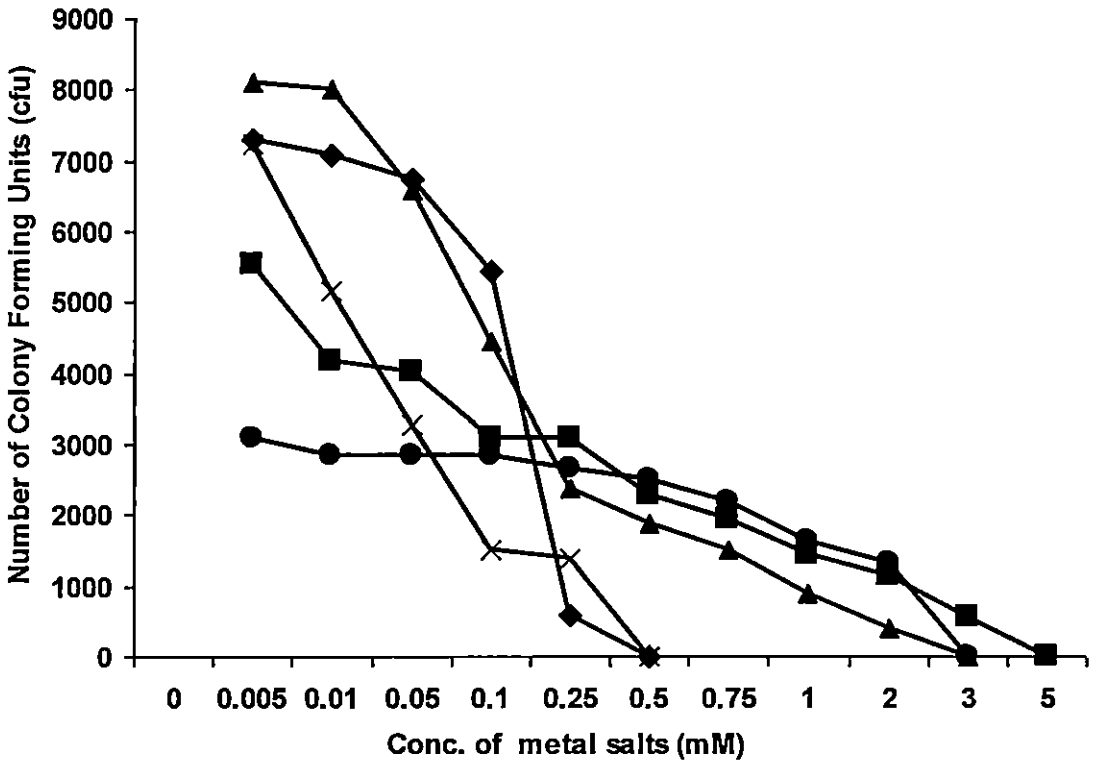

Fig 4.6. Effect of different heavy metal salts on growth of *Frankia* strain (CeSi5) isolated from *C. equisetifolia;* ; ¬ ♦- nickel chloride, ●-cadmium chloride, ▲-cobalt chloride, ■-lead nitrate and X-copper sulphate (Bose and Sen, 2006).

Frankia colonies were re-isolated from various plates and preserved for further studies. Each experiment was repeated twice and was found to produce similar results.

A characteristic heavy metal induced either purple or red or both type of pigment production was noted in all the cases except in lead at MIC or higher concentrations. This type of pigment production is totally different from the pigment production of the strains isolated from *C. equisetifolia* growing in North Bengal University campus (Bose and Sen, 2005, 2006) where a faint red pigment was produced only in lead plates.

When these two results were compared it was found that *Frankia* strain isolated from *A. nepalensis* were less resistant to the heavy metal salts. It was hypothesized that the *Frankia* isolated from *A. nepalensis* facing less heavy metal stress than *Frankia* isolated from *C. equisetifolia.* As the latter was the inhabitant of coastal region which was facing the more metal concentrations due to metal pollution and become more resistant to heavy metal ions. On the other hand hilly forest region of the Darjeeling district is relatively free from metal pollution and hence native *Frankia* were less exposed to heavy metal ions. One of the strain AnMr1

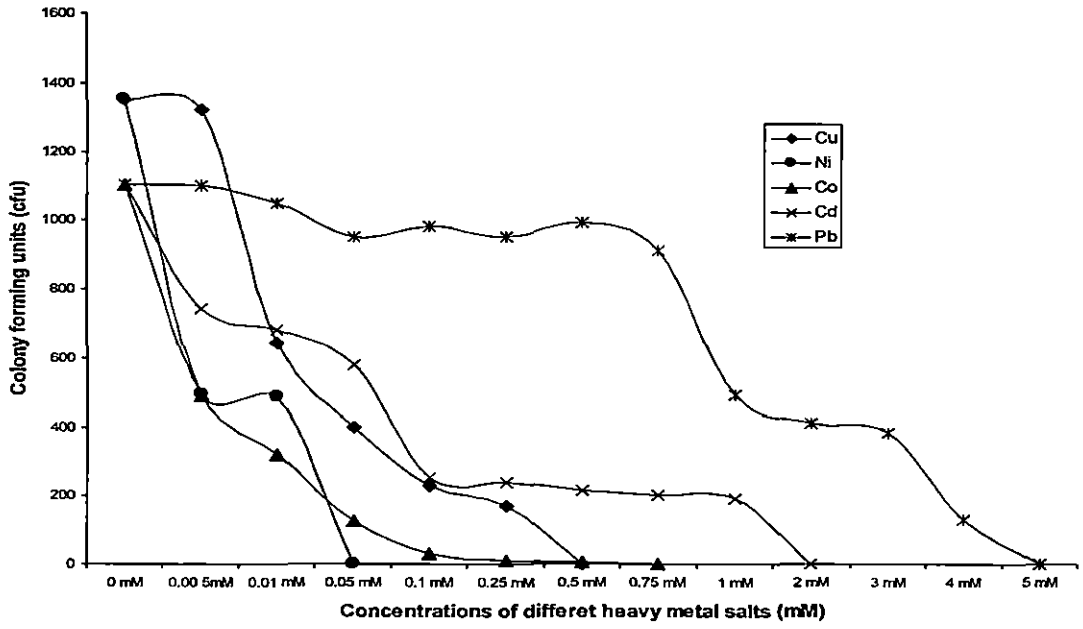

Fig 4.7: Heavy metal resistance pattern of *Frankia* strain (AnMr1) isolated from *A. nepalensis*. (Bose and Sen, 2007).

was found compatible with elevated concentration of cadmium and lead salts.

This had great significance since the heavy metal resistance is a more suitable marker for slow growing bacteria like *Frankia* as it is more stable than antibiotics resistance (Richards *et al.*, 2002).

4.9 Analysis of Heavy Metal Resistance Genes of *Frankia*

The heavy metal resistant genes of *Frankia* are listed in the Tab 4.4. Figure 4.8 showes the dendrogram obtained by the multiple sequence alignment of the studied genes of the three strains of *Frankia*. It was seen from the dendrogram that there was a single major clade and two subclades. The tellurite and tellurium resistant genes clustered together and the copper genes clustered together with the exception of one copper resistance gene from *Frankia* CcI3. Clustering of copper resistance and tellurite resistance genes of the different *Frankia* strains suggests that they have co-evolved as a unit. The Zn-Co-Cad resistance gene had a completely different origin and did not lie in a particular clade. (Bose *et al.*, 2007).

The heterogenecity of the codon usage was significantly related to the translation of that particular gene. The highly expressed genes, like ribosomal protein genes some have high degree of codon bias, depending on their effective number of codon (Nc)

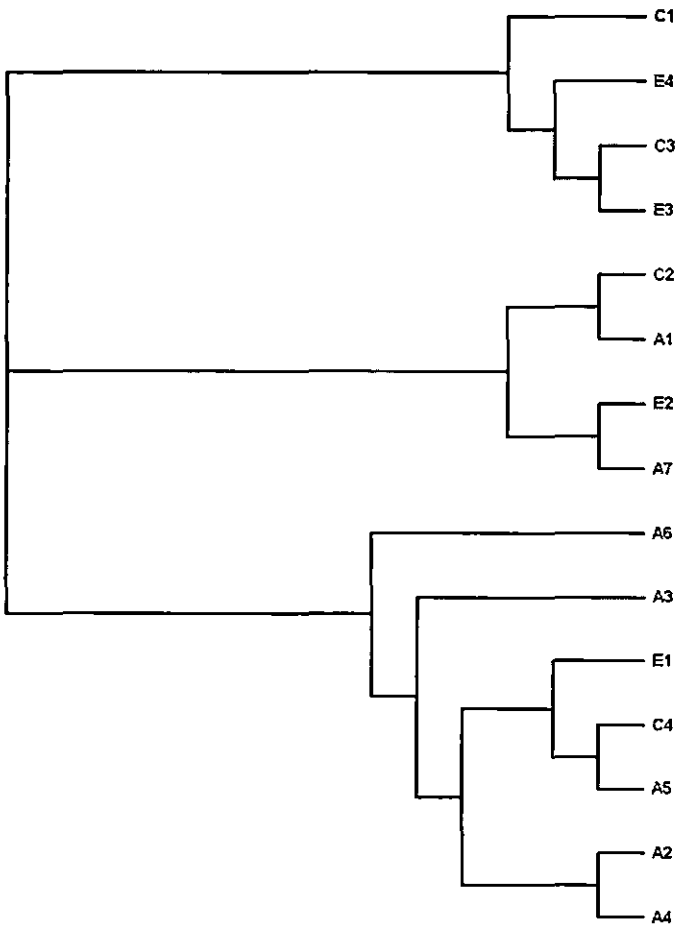

Fig 4.8: Dendrogram of the relationship of heavy metal resistance genes of the *Frankia* strains.

(Ikemura 1981; Lafay *et al.*, 2000). Thus Nc was plotted against the GC3 to investigate the diversity of codon usages in the genomes (Peden, 1999). It is a common fact that high GC containing organism show considerable codon usages diversity (Wu *et al.*, 2005). The ribosomal protein genes were considered as a reference in this work. These proteins were clustered at the lower end of the graph which was an indication of strong codon bias. The heavy metal resistance genes also clustered along the ribosomal protein genes and a strong codon bias was indicated in these cases. The *Frankia* genome showed low value of effective number of codon (<40).

The values of the codon usage indices calculated for the studied strains are shown in Table 4.4. From the results it was seen that the G+C content of the *Frankia* strains were high. Due to this property, it was hoped that the GC3 content and the Nc values for the genes from *Frankia* genomes might have some degree of heterogeneity. It was seen that the Nc values decrease with increase in GC3 content indicating that codon bias increases with the increase in GC3 content. The Nc/GC3 plot demonstrated an effective technique for

Tab 4.3: MIC and MTC of various heavy metal salts

	AnMr1					CeSi5				
Heavy metals used	Copper	Nickel	Cobalt	Cadmium	Lead	Copper	Nickel	Cobalt	Cadmium	Lead
MIC	0.01	0.005	0.005	0.005	1	0.05	0.05	3	5	3
MTC	<0.01	<0.005	<0.005	<0.005	<0.75	<0.05	<0.05	<3.00	<5.00	<3.00
Pigment production	Red	Red and purple	Red	Red	Absent	Absent	Absent	Absent	Absent	Reddish white

investigating codon usage variations among the genes. The Nc value of the genes showed a range for all the genomes suggesting that these highly GC rich genomes exhibited considerable amount of heterogeneity in codon usage. Genes coding ribosomal proteins that are known to be highly expressed were highlighted in the NC/GC3 plots. Compared to the ribosomal protein genes the heavy metal resistance genes in the genomes showed some difference. Heavy metal resistance genes of CcI3 were less biased compared to the ACN14a and EAN1pec strains. Most of the ribosomal protein genes for all the *Frankia* genomes were found to be clustered at lower ends of the plot suggesting a strong codon bias for these genes. However, heavy metal resistance genes of CcI3, ACN14a and EAN1pec strains were clustered at lower ends of the plot (Fig 4.9A,B,C). Genes with low Nc (value <40) have much stronger codon bias than be simply explained in terms of mutational bias. Ribosomal protein genes and those associated with metal resistance had a lower mean Nc value than that obtained for all of the protein coding genes suggesting a higher degree of bias in the former. These

values indicate a strong codon bias over mutational bias. This codon bias arise due to natural selection for translational efficiency (Peden, 1999)

The Codon adaptation index (CAI) assesses the extent to which selection has been effective in molding the pattern of codon usage. Heavy metal genes and ribosomal protein genes had higher mean CAI values compared to that of the protein coding genes. High CAI values indicate better expression levels. Especially the comparatively higher expression levels for heavy metal resistance indicate the ability of *Frankia* genomes to survive in stressed environments. The result obtained in this study put an additional support to the hypothesis that *Frankia* strain CcI3 is more symbiotic than saprophytic, since they remain largely in the nodules (a protective place for them) and hardly grow in the soil.

4.10 Isolation of DNA

The *Frankia* DNA was isolated using the method of Bajwa (2004). The DNA was precipitated forming DNA-CTAB complex as whitish mucus at the bottom of the microcentrifuge tubes. These precipitations were dissolved in 1X TE buffer and used for further analysis. The 0.8% agarose in agarose gel electrophoresis showed good

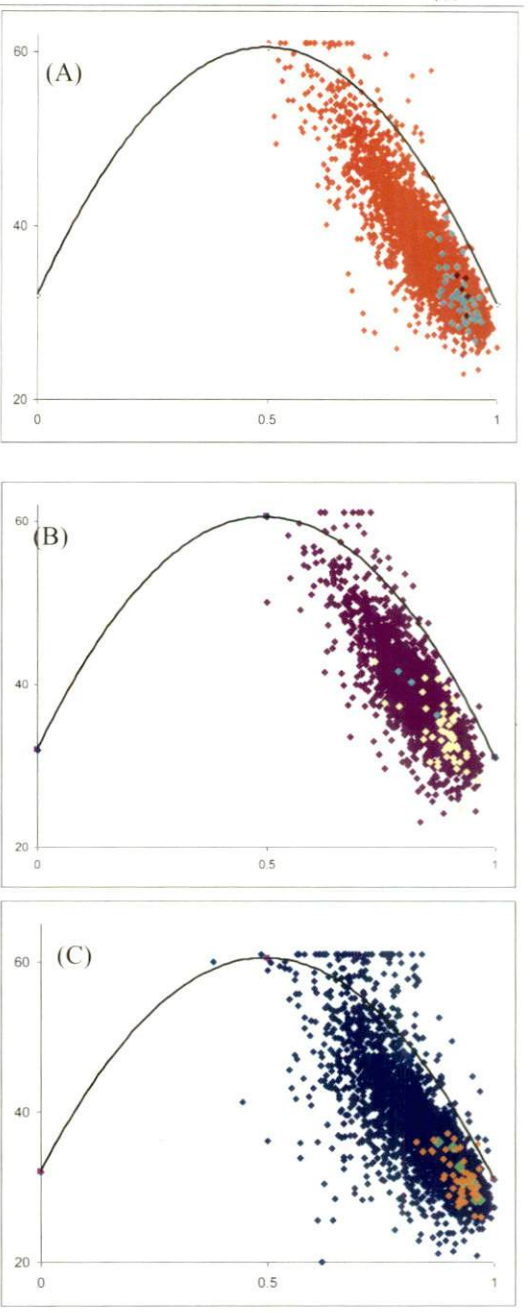

Fig 4.9 A: The NcGC3 curve of *Frankia* Strain EAN1pac: Protein coding gene: Orange; Ribosome protein gene: Green; Metal resistant genes: Pink; B : The NcGC3 curve of *Frankia* Strain CcI3: Protein coding gene: Pink; Ribosome protein gene: Yellow; Metal resistant genes: Green; C: . The NcGC3 curve of *Frankia* Strain ACN14a: Protein coding gene: Blue; Ribosome protein gene: Yellow; Metal resistant genes: Green

bands..

4.11 Purification of DNA

The genomic DNA is generally contaminated by various proteins, RNA and polysaccharides. During the DNA extraction process the proteins were eliminated by phenol and chloroform. The RNA were removed by RNaseA treatment. The polysaccharides were removed by the standard DNA extraction protocol (Bajwa, 2004). To get rid of extra phenolics which in turn hinders the downstream reactions the samples were further purified with glass milk, PEG8000 and sigma kit. These purified DNA was used for polymerase chain reaction. The purified DNA was subjected to spectrophotometric quality testing. All the samples were found to be qualitatively pure with a ratio of A_{260}/ A_{280} of ~1.8.

4.12 Development of A New 16s Primer Sequence for *Frankia*

A 20 bp long primer was developed by

Table 4.4- Values of Different Codon usages Indices for the Heavy Metal Resistance Genes of *Frankia*

Genes	Gene ID	Abbn.	CAI	CBI	Fop	Nc	GC3	GC
Copper resistance D [Ccl3]	637880116	C1	0.228	0.241	0.54	35.2	0.89	0.72
Copper resistance protein CopC [Ccl3]	637880115	C2	0.251	0.203	0.53	38.8	0.82	0.74
Tellurite resistance protein TerB [Ccl3]	637880329	C3	0.334	0.226	0.56	33.3	0.91	0.64
Putative tellurium resistance protein TerA [Ccl3]	637881001	C4	0.342	0.357	0.63	33.4	0.91	0.69
Tellurium resistance protein TerA [Ean1pec]	641239986	E1	0.332	0.369	0.63	29.7	0.94	0.71
Copper resistance protein CopC [Ean1pec]	641246078	E2	0.26	0.327	0.6	32.8	0.92	0.74
Tellurite resistance TerB [Ean1pec]	641240861	E3	0.207	0.197	0.54	36.6	0.86	0.7
Tellurite resistance TerB [Ean1pec]	641241347	E4	0.195	0.192	0.52	34.4	0.91	0.77
Putative copper resistance protein [ACN14a]	638100993	A1	0.229	0.219	0.53	38.9	0.81	0.69
Tellurium resistance protein [ACN14a]	638101739	A2	0.327	0.331	0.62	28.3	0.96	0.68
Tellurium resistance protein terE [ACN14a]	638098466	A3	0.13	0.168	0.49	49.9	0.55	0.77
Tellurium resistance protein terE [ACN14a]	638102547	A4	0.282	0.168	0.5	42.4	0.68	0.67
Tellurium resistance protein terA [ACN14a]	638102544	A5	0.246	0.165	0.49	38	0.7	0.71
Putative Cobalt-zinc-cadmium resistance protein [ACN14a]	638100325	A6	0.264	0.288	0.57	30.7	0.95	0.75
Putative Copper resistance domain [ACN14a]	638096856	A7	0.204	0.124	0.49	35.3	0.91	0.77

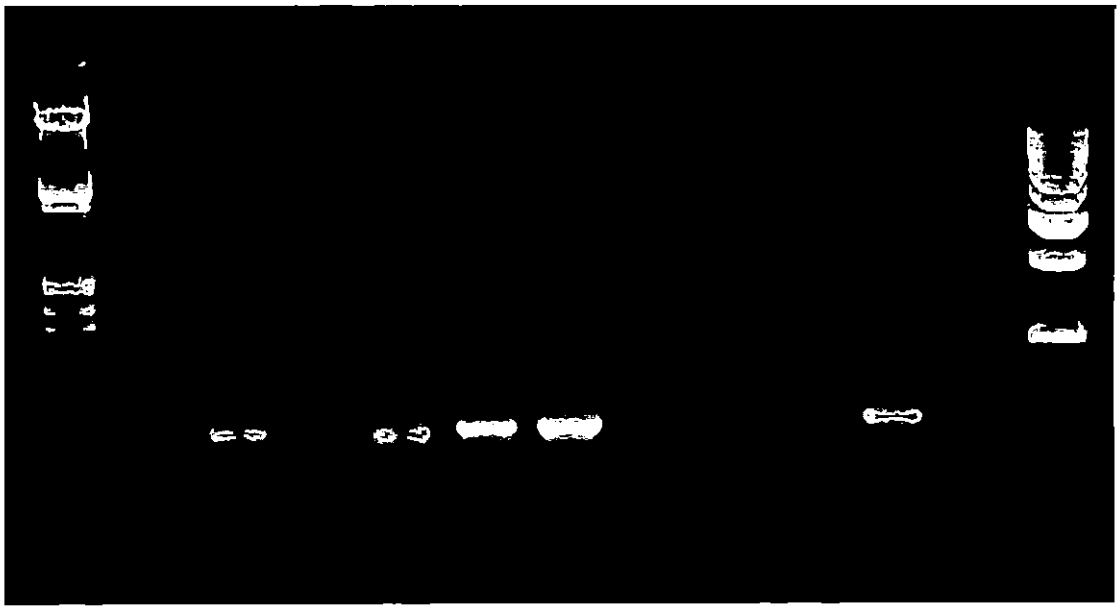

Fig 4.10 Agarose gel photograph of PCR amplification of *Frankia* DNA; Lane1, Lambda DNA *EcoRI/ Hind III* double digest DNA marker; Lane2, –ve control; Lane3-7, A1-A5; Lane8-12, C6-C10; Lane13, 1kb DNA marker (for details refer section No. 3.9.2 of MM).

Primer3. The newly designed primer set could amplify a product of 520 bp from the distal part of 16s rDNA portion of *Frankia* infecting both *A. nepalensis* and *C. equisetifolia* . The sequence was as follows:

forward-5'-GGGTCCGTAAGGGTC-3' and
reverse-5'-AAGGAGGTGATCCAGCCGCA-3'

4.13 PCR Amplification of *Frankia* DNA

The distal part of the 16s rDNA of *Frankia* was the target sequence of the newly developed primer. The primer pair then amplified a single DNA band of expected length (520bp). The PCR products were subjected to restriction digestion with various restriction enzymes, which includes *Alu*I, *Taq* I, *Hae* III, *Mbo*I and *Msp*I. Relatively low level of polymorphism was found.

4.14 Analysis of PCR-RFLP Data

The above results were analyzed with various statistical methods of POPGENE package as mentioned earlier. Among the *Frankia* population the total genetic diversity had been found to be 0.2689 (SD± 0.1858), which showed low level of genetic diversity. This low level of diversity indicates that total genetic diversity comes from inter population source and in this case, there were no lateral gene transfer occur in *Frankia* strains.

Shannon index is used to study the diversity of the population. It is assumed that all species are represented in the sample and they are randomly sampled. The advantage of this index is that it takes into account the number of species and the evenness of the species. The index is increased either by having additional unique species or by having greater species

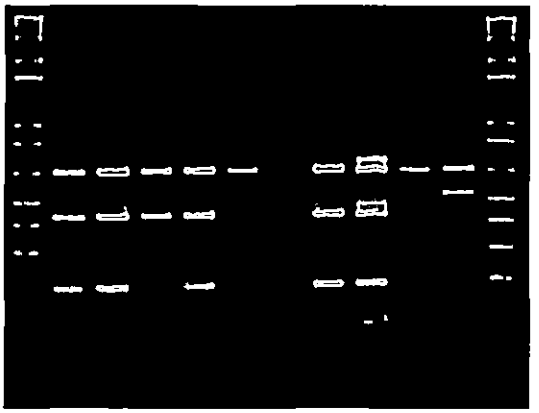

Fig 4.11a. PCR product digested with *Alu*I; Lane 1and 13- 50bp DNA ladder; Lane 2-6, A1 -A5; Lane 7-11, C1-C5. (for details refer section No. 3.9.2 of MM)

evenness. The value of Shannon index will be maximum if the evenness present in the population is maximum. It has specific importance in the expression of equal proportion character in the sample rather than the total number of individuals. The Shannon index has moderate sensitivity towards the sample size. In the present study the value of Shannon index has been found as 0.4062 (SD= ± 0.2619). This value also indicated the low level of genetic diversity.

Thus from the result of statistical analysis, it could be concluded that the *Frankia* population present in this area shows very low genetic diversity and the total genetic diversity was intra population in origin.

The dendrogram based on Nei's (1972) Genetic distance (Method = UPGMA Modified from NEIGHBOR procedure of PHYLIP Version 3.5) (Fig 4.12) showed that the *Frankia* population of *Alnus* based and *Casuarina* based were divided into three major clades. In the first clade *Alnus* based germplasm collected from Mirik (A1), Sonada (A5) and loosely Fatak (A2) were there. These places are within the geographic regionof 10-20 km. In the second clade, we have *Alnus* based Sukhiapukhuri (A3) and Ghoom (A4) which are also geographically close. Among the *Casuarina* based population we found them scattered in different clades. *Casuarina* nodules collected from Diamondharbor (C3) went with *Alnus* nodules of Mirik (A1) and Sonada (A5) where as *Casuarina* based *Frankia* of Bakkhali (C4) were clubbing with *Alnus* based *Frankia* of Sukhiapukhuri (A3) and Ghoom (A4). On the other hand *Casuarina* based *Frankia* collected from Jalpaiguri (C1), Digha (C5) and North Bengal

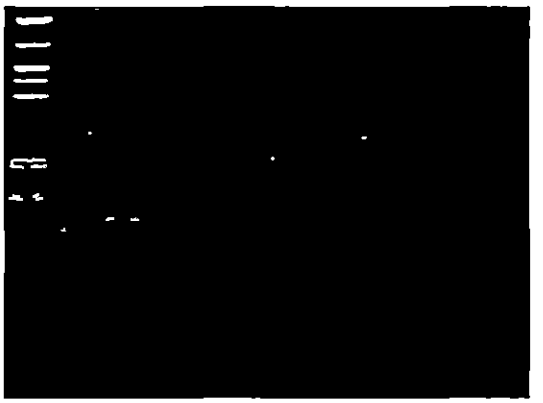

Fig 4.11b. PCR product digested with *Hae*III; Lane 1, 50bp DNA ladder; Lane2-6, A1-A5; Lane 7-11, C1-C5. (for details refer section No. 3.9.2 of MM)

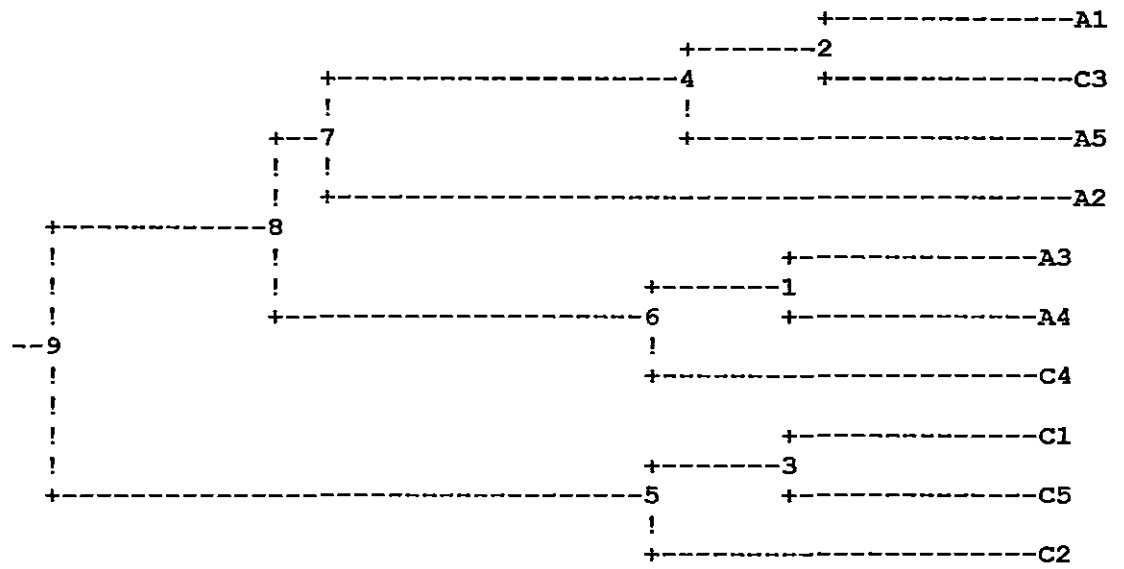

Fig 4.12 Dendrogram Based Nei's (1972) Genetic distance: Method diversity of *Frankia* based on PCR-RFLP results= UPGMA, Modified from NEIGHBOR procedure of PHYLIP Version 3.5

University Campus (C2) comprised of third clade (Fig 4.12).

While from the above result a more or less distinguished pattern emerged in case of *Alnus* based *Frankia*. The *Casuarina* based *Frankia* were places in different clades. The probable reason of *Casuarina* based *Frankia* behaving differently from *Alnus* based *Frankia* could be that all the *Casuarina* plantations in West Bengal are exotic and were perhaps brought from different places.