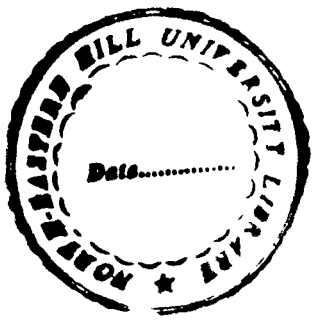
ELECTRON MICROSCOPY AND MOLECULAR BIOLOGY OF *FRANKIA*

By

Arnab Sen



THESIS

Submitted in Fulfilment of the Degree of
Doctor of Philosophy in Botany

NORTH-EASTERN HILL UNIVERSITY

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Thesis

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This work is
dedicated to
the memory of
my father
Shri Paresh Chandra Sen
(1940–1994)

—NORTH-EASTERN HILL UNIVERSITY—

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C E R T I F I C A T E

I certify that the thesis entitled, "Electron microscopy and molecular biology of *Frankia*" submitted by Mr. Arnab Sen for award of Ph.D. degree of the North-Eastern Hill University, embodies the record of the original investigation carried out by him under my supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of Ph.D. degree. The work has not been submitted for any degree of this or any other University.

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STATEMENT

I submit this thesis for the degree of Doctor of Philosophy (in Botany) of North-Eastern Hill University. I declare that this thesis records details of experiments carried out by me and is of my own composition and has not been previously accepted in part or whole for a higher degree of this or any other University.

Arnab Sen

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CONTENTS

Chapter 1

INTRODUCTION

In 1886 Hellriegel and Welfarth announced in Berlin that peas bearing root nodules utilized atmospheric nitrogen for their growth, whereas nonnodulated peas, cereals and other plants could not do so. This discovery settled many aspects of a long running controversy about the sources of nitrogen for the growth of plants and opened up new frontiers of plant and microbial sciences.

The complex process of "fixing" the relatively inert molecular nitrogen i.e. its conversion into reactive forms by the bacteria that inhabit legume root nodules and by many other microorganisms, both symbiotic as well as free living, has been examined in ever increasing details by biologists, chemists, biochemists and biophysicists and is being exploited in modern agriculture. More recently powerful techniques of molecular genetics have also been used for these investigations.

Biological nitrogen fixation is a unique property of some microorganisms. There are no confirmed reports of nitrogen fixa-

tion by eukaryotes except *Eriophorum vaginatum*, an arctic sedge, which uses preferentially the organic nitrogen for growth (ChapinIII *et al.*, 1993).

Nitrogen fixing microorganisms can be divided mainly into two groups, the free living N_2 fixers and symbiotic N_2 fixers. Among the symbiotic nitrogen fixers, the actinomycete belonging to the genus *Frankia* (Goodfellow & Cross, 1984) are of special significance because of their ability to make association with a large number of genera of woody dicotyledonous plants called the actinorhizal plants (Torrey & Tjepkema, 1979). So far 194 species of actinorhizal plants belonging to at least 24 genera spread over 8 dicotyledonous families such as Betulaceae, Casuarinaceae etc., are known to harbour *Frankia* as a symbiont (Benson & Silvester, 1993).

Actinorhizal plants rival legumes in the amount of nitrogen that they fix on a global basis (Schwintzer & Tjepkema, 1990), yet knowledge of their biology and uses is for the most part very recent. They have in common a predilection to grow in marginally

fertile soils and they often serve as pioneer species early in successional plant community development. Representatives can be found in most climatic zones and they inhabit a variety of ecosystems (Benson & Silvester, 1993).

Because they often thrive on marginal soils, actinorhizal plants have current and potential applications in reclaiming and conditioning soils, producing timber and pulp and acting as nurse, windbreak, ornamental and fuel wood plants (Dawson, 1990, Diem & Dommergues, 1990). Globally they have potential for integrating into schemes for addressing issues of pyrodenitrification (Crutzen & Andreae, 1990) and reforestation (Diem & Dommergues, 1990).

The identity of the actinorhizal root nodule endophyte as actinomycete was established in 1964, when electron microscopy revealed the prokaryotic structure of the microorganism in *Alnus glutinosa* and *Myrica cerifera* root nodules (Becking *et al.*,1964). A more detailed description became available only in 1978, when the isolation of strain CpI1 (now known as HFPCpI1) from *Compto-*

nia peregrina nodules was reported (Callaham *et al.*, 1978) and the ability of the organism to reinfect the host plant and re-establish the symbiosis was confirmed (Lalonde, 1978).

Frankia strains are readily recognized by their appearance in liquid culture. They form extensive hyphae and bear sporangiospores in multilocular sporangia located terminally or in an intercalary position on the hyphae. Aerial hyphae are not produced on solid media. A distinguishing characteristic feature of *Frankia* strains is the differentiation of vesicles in culture and some times in symbiosis. Vesicles are lipid-encapsulated, roughly spherical structures measuring 2-6 µm in diameter. They are attached terminally or laterally to hyphae by a short stalk that also is encapsulated. They normally appear in response to nitrogen deprivation. *Frankia* also bears intrahyphal or terminal sporangia of considerable size (up to 60 µm) and complexity containing numerous spores (1-2 µm diameter).

Micro flora in the rhizosphere are of economic importance to agriculture especially in the tropics since they can be used to improve plant productivity and at the same time reduce the need

for fertilizers and chemical pesticides (Okon and Hadar. 1987). Better late than never, efforts are on to exploit *Frankia* to improve soil fertility (Diem *et al.*,1989). In this context, a relatively recent and advanced technique is the polymer entrapment of *Frankia* cells. In the 70's Dommergues group suggested the use of polymer gels to entrap beneficial microorganisms for agricultural inoculants (Dommergues *et al.*, 1979). A decade ago, inoculants made of polymer-entrapped microorganisms were initially tested on *Rhizobium*. The concept is now successfully utilized on an experimental scale on other microorganisms including *Frankia*. A range of substances can be used for the immobilization of cells. These include, polyacrylamide, alginate, xanthan, and a number of other gels such as carrageenan. Frioni *et al.* (1994) observed growth of *Frankia* in alginate beads not only in nutrient medium but in air-incubated condition also, whereas, Borthakur *et al.* (1996) observed germination of spores in to active colonies when incubated even in nitrogen free medium.

Early studies on the molecular biology of *Frankia* were hampered by difficulties with growing enough cellular material for DNA extraction and also by the low efficiency of classical lysis techniques of *Frankia* cells. The Lyon group tried a number of different DNA isolation procedures exploiting such enzymes as lysozyme, protease etc. without much effect on lysing a significant portion of cells. Nevertheless, the use of lysozyme in combination with a drastic extraction method allowed a small amount of DNA. However, the use of achromopeptidase by Simonet *et al.* (1984) gave better results.

The DNA base composition i.e. the proportion of Guanine and Cytosine residues, has been determined. An *et al.* (1983) estimated the G+C percentage of twelve *Frankia* strains isolated from widely different host plants. They found their G+C% to lie between 68-72% depending on which method was used. These results established *Frankia* as a typical actinomycete, since this microorganism has generally a high G+C%. Mullin *et al.* (1983) were able to isolate pure endophyte DNA directly from the nodule tissues. As expected

for DNA with such a high G+C%, restriction endonucleases such as *Sst*I, *Sph*I and *BamH*I that have a high G+C% recognition sites, cut the *Frankia* DNA much more than *Eco*RI or *Bgl*II with a low G+C recognition sites.

PCR based technologies to identify *Frankia* using *Frankia* specific probes were mainly developed by the Lyon group (Simonet *et al.*, 1991). Amplification of specific segments of *Frankia* DNA directly from the nodule via PCR is also available (Misra *et al.*, 1991). A number of RFLP experiments involving a set of restriction endonucleases in the *rrn* region and *nif* region of *Frankia* genomic DNA reveal five different groups of *Casuarina* infective *Frankia* in Australia but surprisingly only one group (Group 1) is found in the rest of the world so far (Rouvier, personal communication). DNA sequences of a number of genes mainly involved in nitrogen fixation are now available (*nif*HDKAB, Simonet *et al.*, 1988; MUllin & An, 1990 and nif*XWZB* and some ORF, Harriott *et al.*, 1995).

Objectives

The present investigations were taken up with the following objectives:

1. Collection of germplasm from different parts of India.
2. Establishing the identity of the isolates using :
   a. nodulation tests
   b. morphological studies
   c. anatomical studies
   d. DNA base composition with respect of G+C mol%
   e. amplification of DNA using *Frankia* specific primers
3. Study of diversity of indian frankiae with respect to morphology, anatomy, nitrogenase activity and PCR/RFLP.

Chapter 2

REVIEW OF LITERATURE

2.1 THE HISTORY OF *FRANKIA* RESEARCH (BEFORE 1978):

The study of root nodules of nonleguminous plants was shrouded in the mist of misconceptions for a long period of time. Meyen in 1829 for the first time gave a description of the root nodules on alder and made the remarkable conclusion that they were parasitic plants growing on the roots. Woronin in 1866 went a step forward by studying the anatomical features of the nodules and found intercellular hyphae passing through the walls from cell to cell. Inside the cells the tips of these hyphae formed round vesicular swellings, which he considered as fungal spores. He named this organism as *Schinzia alni* because of its resemblance to the parasitic fungus *Schinzia cellulicola*.

Frank dismissed the idea of the presence of microorganisms in the root nodules of both legumes as well as nonlegumes by considering them as protein bodies. But Brunchorst (1885) (see Quispel, 1990) a student of Frank, became convinced, studying the differences between the cytology of leguminous and actinorhizal root nodules, that a microorganism was indeed involved in later

ones. He named it *Frankia subtilis*.

In the mean time, between 1886 and 1888 (see Quispel, 1988), Hellrigel and Wilfarth published two papers, which brought an end to a 10 year old controversy about the sources of nitrogen for the growth of plants and opened up new frontiers of plant microbial sciences. Greatest contribution of these two authors was to show that legume nodules fixed atmospheric nitrogen. They also gave an idea about nodule inducing "ferment", showed that it was more or less specific, was killed by moderate heat and harmed by drought. Nitrogen fixed in a nodulated legume was not immediately available to the neighboring plants and that small quantities of combined nitrogen did not effect nodulation whereas larger amount was inhibitory (Quispel, 1988).

These classic experiments of Hellrigel and Wilfarth gave the idea of the difference between nitrogen user and nitrogen accumulator and alders were pushed into the nitrogen accumulating group. But the year long experiment of Hiltner (1904) proved that

actinorhizal plants could utilize the atmospheric nitrogen. As a result, a comparison between leguminous and actinorhizal microsymbiont started which led to the conclusion that the actinorhizal microsymbionts were more bacteria than fungi.

Meanwhile, the bacteria of the legume root nodules had been isolated by Beyerinck. These bacteria failed to infect the nonleguminous plants. The non-infectivity as well as the difference in the cytology led the scientists to consider the two microsymbionts as two different types of microorganisms.

However, as far as the identity of the microorganism was concerned, it was through the classic electron microscopy of Becking and others that presence of an actinomycete was established. A more detailed morphological study of *Frankia* became available only after the isolation of *Frankia* in pure culture by Torrey group (Callaham *et al.*, 1978) which was the most important event in the history of *Frankia* research.

2.2 THE HOST PLANT

Since the genus *Frankia* is an actinomycete, the roots infected by *Frankia* were for the first time named actinorhiza in the first international conference at Harvard Forest in Petersham, Massachusetts held in April 1978 (Tjepkema and Torrey, 1979; Newcomb and Wood, 1987) (for more details see appendix I). The plants which bear the actinorhiza are called as "actinorhizal plants." It should be noted that though the term actinorhizal is analogous to mycorrhizal, actinorhizal is spelled with one "r" (Baker and Schwintzer, 1990).

Unlike *Rhizobium*, which can infect only the members of the family Leguminoseae (exception: *Parasponia*, Ulmaceae), *Frankia* can make symbiotic association with a large number of woody dicotyledons.

So far, 288 species of 24 genera belonging to 8 families of 7 orders have been reported to have actinorhizal associations (Baker and Schwintzer, 1990) (Table 2.1). However, according to Benson and Silvester (1993), there are only 194 species of 24

TABLE 2.1

Taxons of Actinorhizal Angiosperms and *Frankia* Isolates[a]

Order	Family	Genus	Number of Species	Isolates[b]
Casuarinales	Casuarinaceae	*Allocasuarina*	54	+,I,E
		Casuarina	16	+,I,E
		Ceuthostoma	2	-
		Gymnostoma	18	+,I,E
Fagales	Betulaceae	*Alnus*	47	+,I,E
Myricales	Myricaceae	*Comptonia*	1	+,I,E
		Myrica	28	+,I,E
Protales	Elaeagnaceae	*Elaeagnus*	38	+,I,E
		Hippophae	2	+,I,E
		Shepherdia	2	+,I,E
Ranunculales	Coriariaceae	*Coriaria*	16	+,N
Rhamnales	Rhamnaceae	*Ceanothus*	31	+,N
		Colletia	4	+,N
		Discaria	5	+,N
		Kentrothamnus	1	-
		Retanilla	2	+,N
		Talguenea	1	+,?
		Trevoa	2	+,N
Rosales	Rosaceae	*Cercocarpus*	4	+,N
		Chamaebatia	1	-
		Cowania	1	+,?
		Drayas	3	-
		Purshia	2	+,N
Violales	Datiscaceae	*Datisca*	2	+,N

[a]Compiled from Bond (1983), Torrey & Berg (1988), Newcomb & Wood (1987), Baker & Schwintzer (1990) and Benson & Silvester (1993).

[b]Symbols: -, isolates not reported; +, isolates obtained; I, infective; N, noninfective; E, effective in fixing N_2; ?, infectiveness unknown or unreported.

Classification based on Cronquist (1988)

genera, but they have not given a detailed account. Similarly, Lechevalier (1994) mentioned about 25 genera without the 25th genus. Perhaps she meant Rubus (Rosaceae). But despite at least two published reports of nodulation in rubus (Bond, 1976; Becking, 1984), Stowers (1985) did not consider it as a truly nodulated species. Normand (personal communication) also agreed with Stowers.

Actinorhizal plants like to grow in marginally fertile soils and representatives can be found in most climatic zones (see Table 2.2). Figure 2.1 shows the distribution of actinorhizal plants in India.

2.2.1 Taxonomy and Evolution of Actinorhizal Plants:

As mentioned above, actinorhizal plants cover a range of woody dicotyledons and the taxonomic relatedness is very less. Actinorhizal genera are found in ancient as well as advanced lineage (Bousquet and Lalonde, 1990). Besides, all the members of a certain family may not be actinorhizal, while all the genera of the family Elaeagnaceae are actinorhizal.

TABLE 2.2

Representatives of Actinorhizal Plants in various Ecosystems*

Ecosystem	Name of the plants
Alpine	*Alnus, Myrica, Elaeagnus, Hippophae.*
Arctic tundra	*Dryas* spp
Chapparal & xeric	*Casuarina, Purshia, Ceanothus, Cercocarpus, Comptonia* & *Cowania* spp
Coastal dunes	*Casuarina, Hippophae, Myrica* & *Elaeagnus* spp
Glacial till	*Alnus* & *Drayas* spp
Riparian	*Alnus* & *Myrica* spp

*Compiled from personal observations and Benson & Silvester (1993)

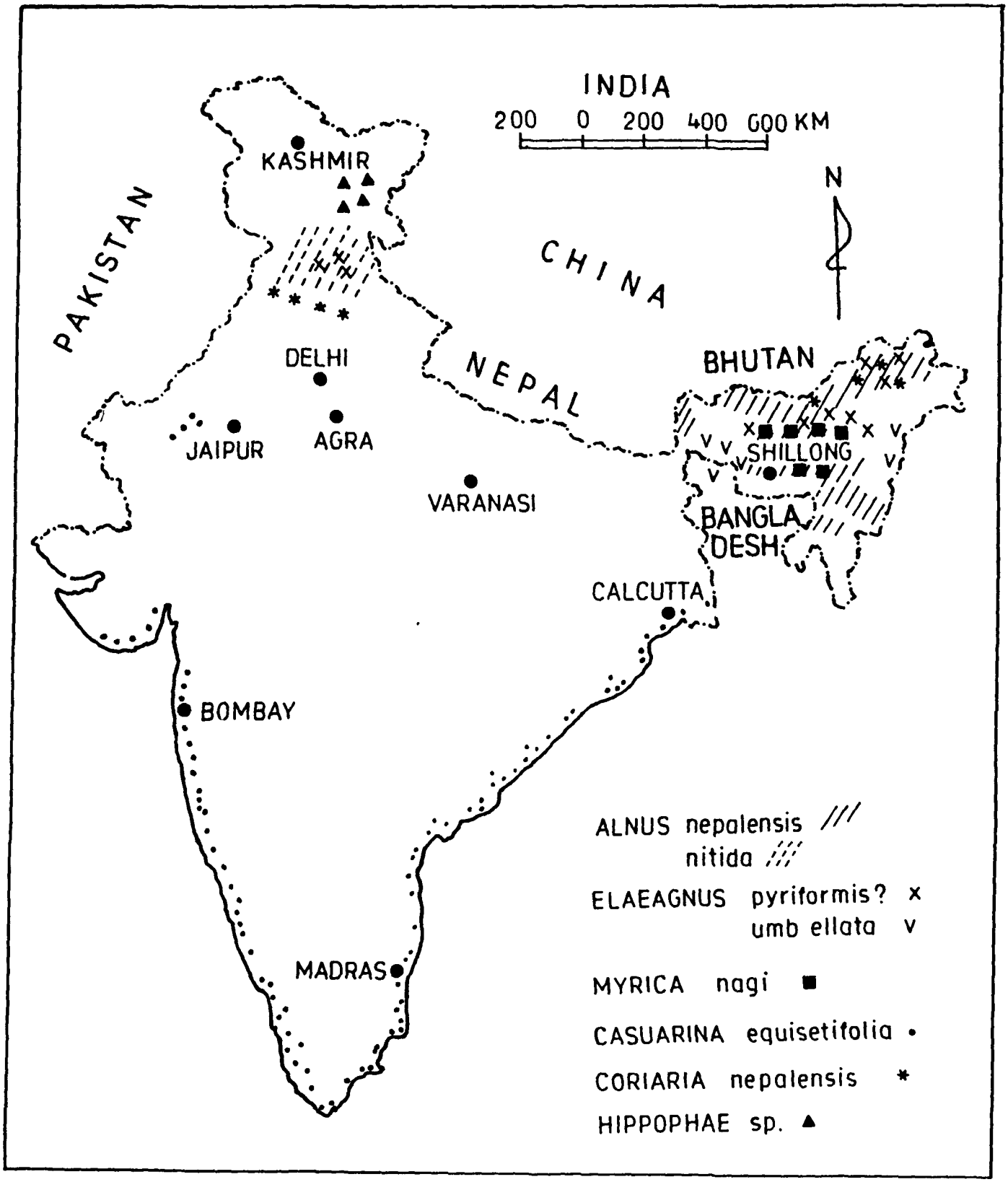

Figure 2.1 Distribution of actinorhizal plants in India

The taxonomic position of actinorhizal genera in the Magnoliopsida (dicotyledons) according to Cronquist (1988) (Fig 2.2) is quite different from the position of Hutchinson's (1973) classification. Particularly in Cronquist's classification, Rosales are not ascribed to a position anterior to the Hamamelidales and the Elaeagnaceae and Rhamnaceae are found in different orders and are shown to have diverged from the Rosales. Datiscaceae are found in the Violales (Dilleniidae) and the Casuarinales are grouped together with Fagales and Myricales in the Hamamelidae. Pollen records show, members of the orders Fagales and Myricales were the first among the actinorhizal plants to visit the earth, while Rosales, Protales and Rhamnales are relatively advanced.

As far as the evolution of the actinorhizal plants is concerned, there are two schools of thoughts. According to the first theory, widely divergent genera acquired the ability to associate with *Frankia* conferring a selective advantage for

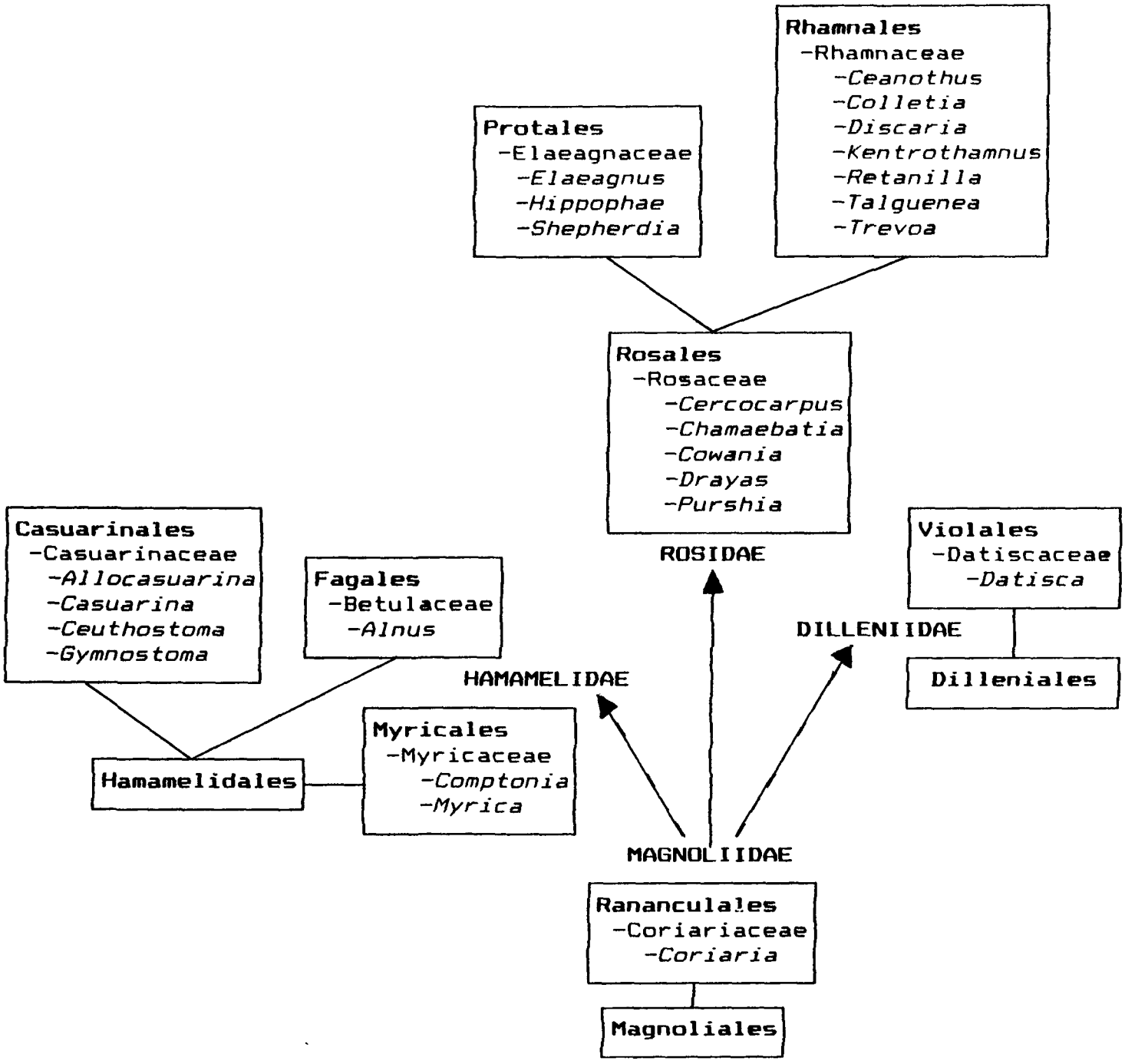

Figure:2.2 Taxonomic position of actinorhizal genera in the Magnoliopsida (dicotyledons) according to Cronquist (1988). Subclasses are shown in bold uppercase, orders in bold lowercase, families in lowercase and genera in italics.

specific ecological niches. So the acquisition of the symbiotic character had hardly taken place before the divergence of families into their respective genera.

The second view assumes the limited availability of nitrogen in the early Cretaceous period (Bond, 1983), forced the then dominant woody wind pollinated angiosperm representatives (e.g. Magnoliidae) to make associations with *Frankia*. However, because of the increase in the availability of nitrogen and decrease in other selective advantages during last 100 M years,the contemporary families lost the symbiotic ability, though the ancestral property to associate with *Frankia* remained as a selective advantage conferred on some specialized early successional plants of nitrogen deficient sites. Study of the phylogeny of *nif*H sequences in *Frankia* (Normand and Bousquet, 1989) gives evidences of the presence of *Frankia* 100 M years ago and supports the second theory. Similarly DNA hybridization studies of host plant supports this hypothesis (Bousquet *et al.*, 1989).

2.3 TAXONOMY OF *FRANKIA*:

Frankia belongs to the family Frankiaceae of the order Actinomycetales. Numerical studies of Hahn *et al.* (1989-a), reverse transcriptase sequencing and oligonucleotide cataloging of 16S rRNA of *Frankia* showed that at least two strains were very much related to *Geodermatophilus* and *Blastococcus*.

Before the successful isolation of *Frankia* in 1978 (see section 2.1), Becking created 10 species of *Frankia* on the basis of host plant specificity, which he determined by using crushed nodules as inocula (Lechevalier, 1994). But after the isolation of various strains, frankial taxonomy became complicated. Especially it has been found that unlike *Rhizobium*, Frankia's host specificity is not very rigid. It has been observed that individual strains often nodulate plants from different plant orders; strains from the same "genomic species" have been isolated from members of different plant families and some strains can not reinfect their source plant (Benson and Silvester, 1993). However, on the basis of phylogeny (Lechevalier

et al., 1983; Lechevalier and Lechevalier, 1989), DNA homology (An *et al.*, 1985) and serology (Baker *et al.*, 1981), *Frankia* has been divided in to two subgeneric groups (Gr.A and Gr.B). The main differences between the groups have been given in Table 2.3.

Akkerman's group (Hahn *et al.*, 1989-b) tried to utilize the hypervariable region of 16S rRNA gene at positions 1020 to 1042 (*E. coli* numbering) for *Frankia* taxonomy at the genus level and found differences comparing the rRNA of effective (N_2 fixing) and ineffective *Frankia* strains. Probes containing 20 to 22 nucleotides targeting this region did not cross react with the rRNAs of a number of actinomycete genera but probes from both effective and ineffective types of *Frankia* reacted with the rRNA of *Nocardioides albus* which decreased the credibility of this method.

Hahn *et al.*(1990) also tried to exploit position 180 to position 240 (*E. coli* numbering) as a probe which was found to react with all the strains tested, but temperature played a significant role, i.e. at higher temperatures some strains did not hybridize which showed their sequence differences. Besides,

TABLE 2.3

Differences between Group A & Group B *Frankia**

Group A	Group B
Organisms are relatively aerobic and most can be maintained on slants.	They are strictly microaerophilic and can not be maintained on slants.
They are physiologically active.	They are physiologically inactive.
They can utilize a variety of monosacharides and disaccharides with or without acid production	They can not utilize carbohydrates, proteins or starch The preferred carbon sources include organic acids or Tween compounds.
Many of them produce hydrolytic enzymes, such as pectinases, cellulases, amylases and proteases.	No such report is available.
At least in the experimental condition, they never reinfect host plant from which they are isolated.	They can infect the host plant from which they were isolated
Their growth is relatively rapid	They grow very slowly (doubling time 2 to 7 days).

*Prepared from Lechevalier (1994).

Actinomadura and *Microbispora* strains reacted positive when tested with other actinomycete genera.

Simonet *et al.*(1991) looked at the problem of genus specific characterization of *Frankia* from a different point of view and tried to solve it by polymerase chain reaction. They designed two sets of primers. The first set was a universal one and targeted nitrogen fixing microbial genera but giving negative results with non nitrogen fixing bacteria. The second set was specific for *nifH-nifD* regions of *Frankia* spp. Then with these two primer sets they amplified DNA of various *Frankia* strains through PCR, which gave rise to genus specific long fragments. Likewise, 16S rRNA gene primers were used to generate fragments whose lengths were specific for the members of the family Frankiaceae (*Frankia* and *Geodermatophilus*). To separate the members of different genera, two additional primers were designed which amplified the 16S and 23S rRNA intergenic sequences which in case of *Frankia* was 561 bp, more or less same for all isolates tested and different from *Geodermatophilus* or other microorganisms.

2.3.1 Taxonomy of the species

Perhaps the most critical part of *Frankia* taxonomy is its speciation. The main difficulties are: (1) individual strains often nodulate plants from different plant orders (Benson and Silvester, 1993), (2) strains from same "genomic species" have been isolated from members of different plant families, (3) some strains failed to reinfect their source plants (Mirza *et al.*, 1991) and (4) unpredictable nodulating abilities have been observed among the *Elaeagnus* strains (An *et al.*, 1985; Fernandez *et al.*, 1989).

Above problems led scientists to try several modern approaches, besides the conventional techniques like morphological differences, *in planta* sporangia formation (Normand and Lalonde, 1982), phylogeny of carbohydrate uptake (Lechevalier *et al.*, 1983; Ganesh *et al.*, 1994), utilization of various substrates and enzyme production (Shipton and Burggraaf, 1982), serology (Baker *et al.*, 1981; Lechevalier *et al.*, 1983), host specificity (Bosco *et al.*, 1992) isozyme patterns (Maggia *et al.*, 1990), whole cell

sugar chemistry (Lechevalier, 1986) etc., with varying degrees of success. The modern approaches include DNA-DNA relatedness analysis, low-frequency restriction fragment analysis (LFRFA), 16S rRNA gene sequencing and PCR-RFLP etc.

An *et al.*(1985) were the pioneers to study the DNA-DNA hybridization and found high level of relatedness among most of the isolates obtained from *Alnus* spp. but failed to find any such relations among isolates of other species, such as *Casuarina*, *Elaeagnus* etc. (Lechevalier, 1994). Fernandez *et al.* (1991) found three genomic species among the 14 isolates of *Alnus*, five genomic species among the isolates from Elaeagnaceae family, and one genomic species comprised nine isolates obtained from the members of Casuarinaceae. The study of DNA sequence of a 274 bp region of an rRNA operon (hypervariable region E_2) of different isolates of *Alnus*, members of Elaeagnaceae and members of Casuarinaceae by Normand and his colleagues (Fernandez *et al.*, 1991) further confirmed the division of isolates into genomic species by DNA-DNA relatedness analysis in case of Alnus. However, they found little

difference in the ribosomal sequences of genomic species of the isolates of Elaeagnaceae.

But when PCR technology was applied to amplify 268 bp DNA segment of the 16S rRNA gene, Nazarat *et al.* (1991) compared all 9 genomic species of Fernandez *et al.*(1989) and found that with one exception, the strains belonging to the same genomic species had identical sequences and they differed from other genomic species.

To measure the phylogenetic relationships, Nazarat *et al.* (1991) developed a protocol based on the amplification and sequencing of 16S ribosomal DNA sequence. They also prepared a phylogenetic tree based on the above mentioned DNA sequence analysis and found that strains belonging to the *Alnus* infectivity group are closely related to strains belonging to the *Casuarina* infectivity group, while *Elaeagnus* infectivity group was well separated from the above two groups.

Another method which gave considerable contribution towards the phylogeny of *Frankia* was restriction fragment length polymorphism (RFLP) of *nif* complex (*nif*AB, *nif*K and *nif*H). Nazarat *et al.* (1989) found highly homologous results in case of infective isolates obtained from *Casuarina* spp. regardless of the restriction enzyme used, whereas non-infective strains differed from the first group and among themselves. However, Akimov and Dobritsa (1992) observed high level of DNA-DNA relatedness among both infective and non-infective isoletes. When Simonet *et al.* (1989) did RFLP analysis of 100 isolates from single alder stand using *nif*HDK probes, they found similarity but when they were probed with *nif*AB, five groups were found.

Jamann *et al.* (1993) amplified intergenic spacer (IGS) and a part of genes in the *nif* cluster through PCR and did RFLP analysis cleaving them with 4 different restriction enzymes and the patterns were used as fingerprints to type *Frankia* strains. They found that the DNAs of *Frankia* isolates obtained from members of the Elaeagnaceae and Casuarinaceae could be amplified to

yield a product of about 1380bp. The DNAs of isolates obtained from *Alnus* spp. were amplified only weakly or were not amplified.

Marry P. Lechevalier and her colleague M. Beyazova employed a novel and simple method recently to tackle the species problem. They used restriction enzymes with less number of restriction sites to cut the DNA into fragments of bigger molecular weight. Then the fragments were separated with the help of pulse field electrophoresis. This method is called as low frequency restriction fragment analysis (LFRFA). The findings of Beyazora and Lechevalier (1992) through LFRFA confirmed the rsults of Nazarat *et al.* (1991) that the type strain of *Frankia alni* subsp *pommerũ* clusters at some distance from the other *Frankia* genomic species isolated from *Alnus* spp.

2.4 *FRANKIA* IN SYMBIOSIS

Frankia form symbiotic association with dicotyledonous plants resulting in the formation of root nodules. In the coming paragraphs the morphology, anatomy and different metabolisms will be discussed.

2.4.1 Morphology and Anatomy

Though the type of symbiosis of both *Rhizobium* and *Frankia* induced root nodules is more or less similar, they differ markedly in development, morphology, and anatomy. While the infected zone of legume nodules central and is normally contained within an endodermis and an inner cortical layer of tightly packed cells, the actinorhizal nodules normally have a central stele that has infected tissue adjacent to it or around it (Benson and Silvester, 1993).

2.4.2 Process of infection

Frankial infection takes place in two different ways in different plants. In case of *Alnus*, *Casuarina*, *Comptonia*, *Myrica* etc. the infection takes place through root hair deformation (Berry and Torrey, 1983; Callaham *et al.*, 1979; Torrey, 1976). While, in case of *Ceanothus*, *Elaeagnus*, *Shepherdia* etc., it takes place by a process of intercellular penetration of root epidermis and cortex (1991; Miller and Baker, 1986; Racette and Torrey, 1989).

In case of root hair infection, though one infection is enough to bring about nodulation (Berry *et al*.. 1986), the frequency of nodule formation is directly proportional to the amount of innoculum (Newcomb and Wood, 1987). It is possible that more than one infected root hair sometimes form one nodule, because, more than one strain have been isolated from single nodule.

Frankia sometimes takes the help of other soil bacteria to deform the host plant root hair. Knowlton *et al.* (1980), Berry and Torrey (1983) & Knowlton and Dawson (1983) reported that in case of *Alnus rubra*, deformation of root hair occurs rather quickly (within few hours) with a helper bacterium such as *Pseudomonas capacia*. It also increases the rate of nodulation, though , its presence is not compulsory for the process.

In *Elaeagnus aungustifolia* and species of *Hippophae*, nodules are more frequent in those regions of root where the hairs are

lacking than the profuse root hair region. It has been found that the entry point of the hyphae appeared to be in the epidermal cells with subsequent hyphal growth occurring in the intercellular spaces between cortical cells. The intercellular spaces contain an electron-dense matrix in which the hyphae grow (Newcomb and Wood, 1987). In *Ceanothus* species intercellular penetration of the epidermis occurs in the presence of root hairs but surprisingly they are not infected (Benson and Silvester, 1993).

The mode of infection entirely depends upon the host plant. It has been found that , both *Elaeagnus* and *Myrica* infective strains or both *Shepherdia* and *Gymnostoma* infective strains enter either by root hair deformation or intercellular penetration depending on their host (Miller and Baker, 1986; Racette and Torrey, 1989).

After entering into the plant root hair, the infective hyphae become encapsulated with a layer of plant cell wall like material surrounded by the host plasmalemma. This encapsulation

is continuous with the host cell wall and surrounds all infective stages both during invasion and in the mature nodule (Lalonde, 1977; Lalonde and Knowles, 1975a; Lalonde and Knowles, 1975b) Immunogold localization and flurescin-conjugated alginate and pectate probes were used to study the biochemical nature of the capsule and it was found that the capsule is made up of cellulose, hemicellulose and pectins (Berg, 1990).

Frankia produces vesicles *in vivo* in most cases (though in *Casuarina* root nodules, usually vesicles are not formed). Vesicles are mainly of three types: (1) Spherical vesicles are found in *Alnus* and *Elaeagnus* Nodules, (2) Members of Rosaceae i.e. *Cercocarpus* and *Dryas* spp have monoseptate elliptical vesicle (We do not know what would be the Nitrogenase protection mechanism in this case), (3) Club shaped hyphal ending which can be called as vesicles are found in the species of *Casuarina* and *Comptonia* (Newcomb and Wood, 1987).

2.5 *FRANKIA* IN PURE CULTURE

In vitro studies of *Frankia* were possible only after the isolation and subsequent culture axenically on yeast extract medium. But improvements in the composition of the culture media now permitted *Frankia* to be grown *in vitro* much faster than on the earlier used yeast-extract medium (Lalonde and Calvert, 1979; Tjepkema *et al.*, 1986).

In their classical reviews Newcomb and Wood (1987) and Benson and Silvester (1993) gave vivid descriptions of the structure and ultrastructure of *Frankia* cells. Benson and Silvester (1993) also talked about the difficulties of the study with the electron microscope because of the delicate nature of some components of the cells. Our personal experience was also like this, in particular the extracellular envelop of vesicles.

2.5.1 Structure of *Frankia* in pure culture

In pure culture *Frankia* is found to behave as microaerophilic and mesophilic microorganism (Burggraaf and

Shipton, 1982). It commonly grows *in vitro* in the form of dense mats of anastomosing hyphae with sporangia developing terminally and at intercalary positions (Newcomb and Wood, 1987) In nitrogen limited conditions it also produces vesicles Structure and ultrastructure of different parts of *Frankia* is given below

2.5.1.1 Hyphae

Under light microscope, *Frankia* show branched septate hyphae. The diameter of hypha is below 0.5 to 1.5 μm. The Havard forest group (Baker *et al.*,1980, Lancelle *et al.*, 1985) as well as Lechevalier and her coworkers (Horriere *et al.*, 1983) have found the *Frankia* cell walls to be composed of two layers of electron dense material, a base layer and an outer layer when fixed chemically.

Benson and Eveleigh (1979) found numerous rosette shaped granular glycogen bodies inside the hyphal cells. Around the periphery of the hyphal cell cytoplasm, large number of cytoplasmic tubules, circular in cross section and averaging 45 nm in diameter, were present (Lancelle *et al.*, 1985). Newcomb *et*

al. (1979) first observed an extracellular multilayered envelop in hyphae of free living *Frankia alni* HFPCpl1 This envelop has been much studied on vesicles but its presence on hyphae is still the subject of debate (Benson and Silvester, 1993).

2.5.1.2 Sporangia

Sporangia are produced readily in culture by all *Frankia* strains isolated (Simonet *et al.*, 1994) and spores as infective propagules are 1000 times more effective at nodule production than are equal volume of hyphae (Burleigh and Torrey, 1980). Ganesh (1993) reported the effect of antibiotics on sporulation and it has been found that medium containing 2 µg/mL of Ampicillin produces profuse sporulation.

In contrast, among *Frankia* strains nodulating the genera *Alnus, Comptonia and Myrica*, a preliminary classification was based on the morphology of the actinomycete inside the nodules. Some strains were described as differentiating many sporangia *in planta* and consequently were called sp^+ strains as opposed to sp^-

strains, which differentiate few or no endophytic sporangia (Schwintzer, 1990). As already mentioned, all *Frankia* sp^- strains isolated to date posses the genetic capability to form sporangia, because when grown *in vitro*, they usually sporulate freely. However, when reinoculated into host plants, they all were unable to differentiate sporangia and thus were defined as sp^- strains. No one has succeeded in isolating in pure culture a typical sp^+ strain (Simonet *et al.*, 1994). In some cases isolation experiments conducted with sp^+ nodules led to the isolation of some *Frankia* strains. These strains, however, failed to differentiate sporangia *in planta* when reinoculated different host species and were thus considered sp^- strains. To explain such a phenomenon, two hypotheses have been proposed. One hypothesis states that physiological conditions (pO_2, soil water content and the age of the plant) determine the *Frankia* endophytic phenotype, i.e. inhibiting or stimulating sporulation. The other hypothesis is that more than one strains coexist in a single nodule. The later hypothesis was proposed because several lines of evidences indicated that endophyte sporulation is

determined by the genotype of the microsymbiont (Schwintzer, 1990; Torrey, 1987). Some nodules classified as formed by sp^+ strains because of the large number of spores obtained *in planta*, might actually contain some particles belonging to a *Frankia* sp^- strain. These rare particles of the sp^-*Frankia* strains, differing from the sp^+ strain by their ability to grow *in vitro*, would develop over the sp^+ strains during the isolation step and would then be routinely propagated *in vivo* (Simonet *et al.*, 1994).

Sporangia develop as terminal or intercalary structures (Newcomb *et al.*, 1979). Segmentation within the enlarging sporangia produces multicellular sporangia containing many spores. Chemically fixed material shows the developing spores with electron translucent nucleoid regions with dispersed fibrils and numerous lipid droplets. Like the hyphae, chemically fixed external walls often show the residue of a laminate envelop (Lalonde *et al.*, 1976). The mature spores show evenly dispersed cytoplasm, but the tubules, which are such a prominent figure of the hyphae are not present in the developed spores (Lancellle *et*

al., 1985). Regarding the arrangement, the more mature spores are at the top and less mature spores are found at the bottom. Sporangia develop by hyphal thickening and then by the formation of septa originating from the inner layer of a double layered sporangial cell wall (Horrier *et al.*, 1983). This type of sporangia has been termed enterothallic by analogy with spore development in fungi (Locci and Sharples, 1984).

2.5.1.3 Vesicles

Frankia differs in many ways from other actinomycetes but the most important difference is the presence of a specialized structure called vesicle. It resembles in many ways with cyanobacterial heterocysts. In fact, vesicles are physiologically isolated from other parts of *Frankia* cells to protect nitrogenase enzyme from oxygen. Normally vesicles are found when *Frankia* are allowed to grow in a nitrogen limited medium or in the presence of certain nitrogen sources such as some amino acids that cannot be digested to ammonia (Benson and Silvester, 1993). Of course, some strains produce vesicles even in the presence of NH_4^+ (Gauthier *et al.*, 1981; Meesters *et al.*, 1985).

In nitrogen limited medium, at the onset of vesicle production, tips of the main hyphae or short side branches swell up. The early structure, which is called as provesicle, is separated by a septa near the base. *Frankia* provesicles are 1.5 to 2.0 μm in diameter and phase dark when examined with phase optics (Newcomb and Wood, 1987). Soon provesicles develop into mature vesicles (2 to 4 μm diameter) which are phase bright. Torrey and Callaham (1982) observed birefringence under polaroid light. Hence, they inferred the presence of a highly structured laminated layer which they showed by elegant freeze fracture techniques to be a laminate of lipid monolayers. These layers appear to be totally lost in normal fixation procedures, leaving a space around the vesicles which is called "void area" (Benson and Silvester, 1993).

2.5.1.4 Protection of Nitrogenase from oxygen

As usual *Frankia* nitrogenase also is O_2 labile. The work of Lyon group (Normand & Bousquet, 1989; Normand *et al.*, 1988 and

Simonet *et al.*, 1986) on nucleotide and amino acid sequence analysis of *nif*H, *nif*D and other *nif* genes confirm the similarity of *Frankia* nitrogenase with the nitrogenase I. Nitrogenase I has two components: dinitrogenase I i.e. component I or MoFe protein, and dinitrogenase reductase I (also called component II or Fe protein). Dinitrogenase reductase I which serves as an electron donor to dinitrogenase I, is a dimer of two identical subunits with a Mr of approximately 60000. A single (4 Fe-4S) cluster is bridged between the two subunits (Gillun *et al.*, 1977). Dinitrogenase is a tetramer with a Mr of about 220000. Tt is made up of two parts of nonidentical subunits (α & β). Dinitrogenase I contains two types of metal centres involved in the redox reactions of the N_2 reduction process: P centres that might be organised as four unusual 4Fe-4S clusters (Eady, 1986; McLean *et al.*, 1988) and two identical FeMo cofactors (FeMoco) that are almost certainly the sites for N_2 binding and reduction (Smith *et al.*, 1985). However, there is no report of the presence of nitrogenase II i.e. vanadium based and nitrogenase III i.e. iron based nitrogenase in *Frankia*. Though, Ganesh (1993) had found

that the medium with vanadium increased reduction of C_2H_2 to C_2H_4, but the study was not very conclusive and further study is needed on this aspect.

The striking feature of *Frankia* over *Rhizobium* is the ability to fix nitrogen in pure culture even when grown aerobically (personal observation). It has been found through a number of studies, that both in symbiosis as well as pure culture, nitrogenase is located in the vesicle (Huss-Danell & Bergman, 1990; Mian & Bond, 1978; Sasakawa *et al.*,1988; Meesters *et al.*, 1990; Noridge & Benson, 1986; Tisa & Ensign, 1987a; Tisa & Ensign, 1987b; Tjepkema *et al.*, 1980).

Chapter 3

MATERIALS AND METHODS

3.1 ISOLATION AND CONFIRMATION OF IDENTITY OF *FRANKIA*

3.1.1 Collection of germplasm

Germplasm was collected from different parts of India. The places included Madras, Rameswaram, Thiruchirapally, Thiruvananthapuram, Coimbatore, Bhubaneswar, Puri, Digha, Siliguri, Itanagar etc. (see also Fig. 3.1).

After going to the site of the vegetation, essential data were collected (for details see Fig-3.2), photographs were taken (plate A) and then the vegetation around the tree of interest was cleared off. The soil was dug to reach the nodulated roots. The depth of the first appearance of nodules varied from 1" to 18". Since several leguminous plants were present near the actinorhizal plants, nodulated roots were traced to the tree of their origin to avoid any confusion about the identity of the nodules. Mostly young and healthy trees were chosen and the young and developing nodules were collected. The percentage of nodulation was calculated by the following formula:

Plate A

Casuarina equisetifolia along the coasts of India. Two different collection sites of *Frankia* germplasm.

$$\text{\%age of nodulation} = \frac{\text{No. of plants with nodules}}{\text{No. of plants observed}} \times 100$$

The collected nodules were then kept in polyethylene bags on ice in an ice box throughout the journey and stored at -20°C in the laboratory.

3.1.2 Isolation of endophyte

3.1.2.1 Surface sterilization

Prior to isolation experiment, the nodules were brought out of the deep freezer, allowed to thaw and brought to room temperature. They were then washed thoroughly with plenty of water. After removal of soil and other foreign particles, nodules were washed with a few drops of detergent. Lastly they were washed with distilled water.

The nodules were collected from the rhizosphere that contained several other microorganism (Plate B-3). Since *Frankia* is a slow growing organism chances of soil born, fast growing, microorganisms contaminating the cultures are more. To overcome

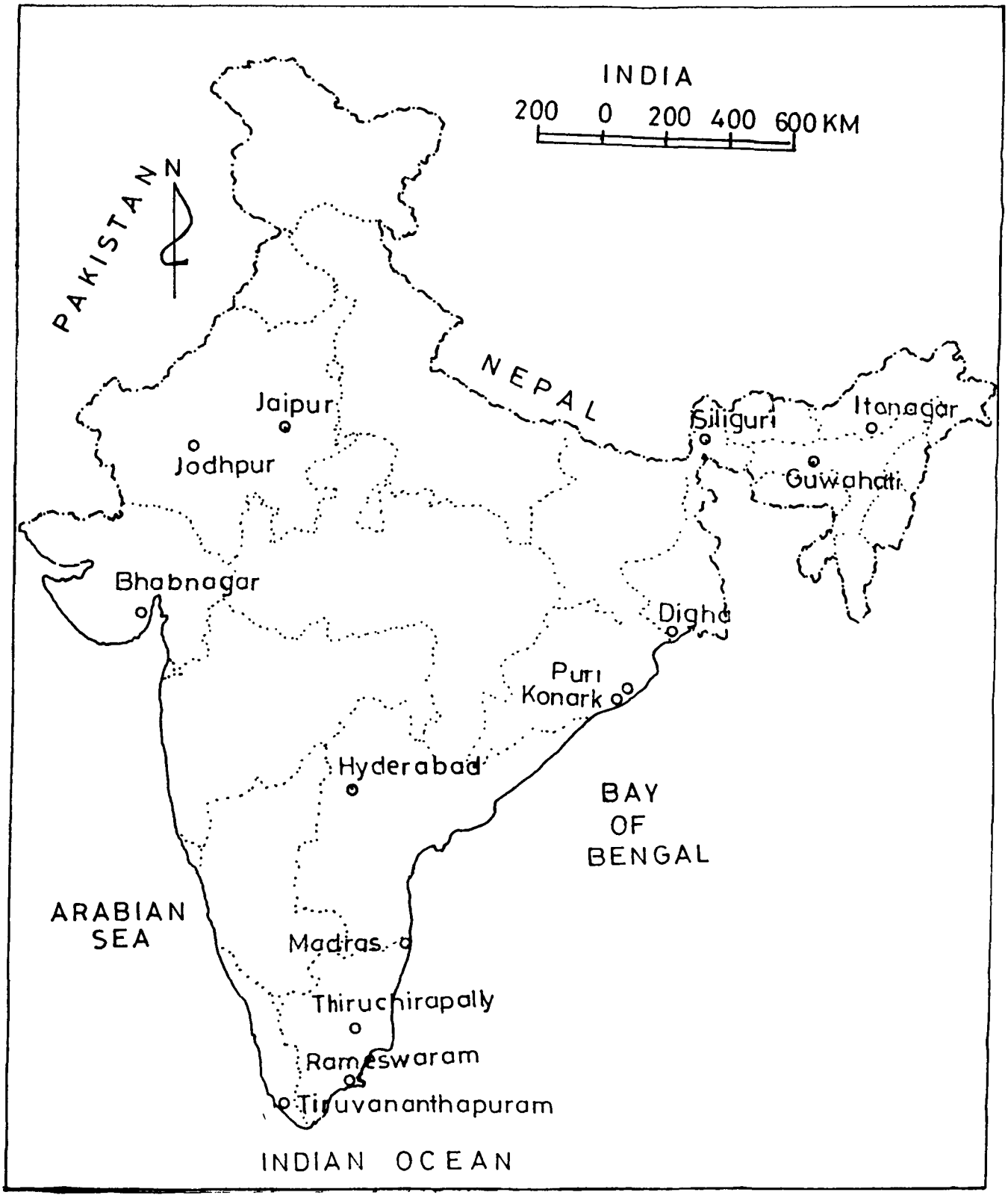

Figure:3.1 Sites of collection of germplasm.

COLLECTION DATA SHEET

SAMPLE NO. D-21

A. GENERAL INFORMATION

1. Collection site & state Digha, W. Bengal
2. Date 28.2.93
3. Local name Thau.
4. Scientific name Casuarina equisetifolia
5. Nodules (present)/absent
6. Nodules collected (Yes)/No
7. Root collected (Yes)/No
8. Seed collected (Yes)/No
9. Soil collected (Yes)/No
10. Twig collected (Yes)/No

B. HABITS

1. (Tree)/shrub/herb
2. Flowering time Jan - March.
3. Seeding time
4. Planting time —

C. HABITAT & AREA OF THE VEGETATION

1. Rainfall Seasons: From April To September
2. Altitude:
3. Topography: Swamp/Plain/Hilly/Mountain/Others Seashore.
4. Veg. Type: Natural Forest/Road side/ (Social Forest)/Others
5. Management: Cutting/Burning/Natural/ Habitat Preserved
6. Associated Species Grass.

D. SPECIFIC COLLECTION SITE

1. Site cover: (Bare)/Up to 20%/21-40%/41-60%/ 61-80%/81-100%

E. SOIL TYPE

1. Soil Texture: (Sandy)/Loamy/Clayey/Organic/Rocky
2. Soil pH 6.2
3. Colour red/(Yellow)/Brown/Grey
4. Drainage: Flooded/poorly drained/(well drained)

F. NODULES

1. Location: crown area/tap root/(lateral root)
2. Growth form: (clumped)/scattered

COLLECTED BY: NAME ARNAB SEN SIGNATURES A Sen 28/2/93

Figure: 3.2 Sample data sheet for collection of actinorhizas in the field.

this problem, surface sterilization of nodules was done very carefully. For this purpose, various methods and chemicals were tried. Some of these are as follows:

1. Nodules were treated with 0.1% $HgCl_2$ for 2 min. followed by washing with sterile distilled water eight to ten times.
2. Nodules were treated with 10% H_2O_2 for 10 minutes in a petridish followed by several washes with sterile distilled water.
3. Nodules ware placed in a side arm flask attached to a pump along with 30% H_2O_2 for 20 minutes followed by several washes with sterile distilled water..

3.1.2.2 Isolation of Frankia *in liquid culture*

After proper surface sterilization, all the isolation techniques described below were tried:

1. Surface sterilized nodule lobes were crushed on the sides of the culture tubes containing liquid growth medium for *Frankia*.

2. Surface sterilized root nodules were chopped in to small pieces and put into liquid medium.

3. Surface sterilized root nodules were incubated at 28-30^{o}C for seven days in Q_{mod} medium. Any viable spores of other organisms still present on the surface, germinated in some cases. These vials were discarded. Where no contamination appeared, nodules were crushed on to the wall of the vials containing liquid media.

4. Sterilized nodule tissues were homogenized in a sterile tissue homogenizer with 3% PVP (Polyvinyl pyrrolidone, SRL, Bombay, India) in PBS (pH 7.4) (see Appendix III for composition). Kept at room temperature for 10 minutes to allow the tissue debris to settle down. Then serial dilution was done.

5. In this technique, the sterilized nodule lobes were processed in the following way:

   a. Each nodule lobe was taken on a sterilized slide.

   b. Upper epidermal layer was peeled off with two sterilized needles.

c. The nodule lobe was washed twice with sterile distilled water.

d. It was then chopped on a sterile slide with a sterile scalpel.

e. The chopped pieces were then inoculated in vials with suitable media.

3.1.2.3 Isolation of Frankia *in alginate beads*

After proper sterilization, about 0.5 g. of nodule lobes were taken in a tissue homogenizer with 10mL of 3% PVP in PBS (for composition and preparation see Appendix III). The nodule lobes were crushed properly and debris was allowed to settle down for 10 min. The upper aqueous phase was then taken in a sterile conical flask and kept overnight in the refrigerator to get rid of the phenolics present in the nodules (Lechevalier & Lechevalier, 1990). The next day, 1g of Na-alginate (Sigma chemical Co. USA for structural formula see appendix IV) was dissolved in 40 mL of liquid medium by keeping it in boiling water bath. Along with this, 250mL of 1% $CaCl_2$ soln. was also

prepared. Both the solutions were sterilized by autoclaving for 20 min. at 1.1kg/sq cm. Then the aqueous phase of crushed nodules was mixed with the Na-alginate solution when the temperature of the solution came down below 30°C. They were then dropped asceptically through a sterile syringe in to the sterile $CaCl_2$ solution and kept half an hour in the refrigerator for hardening. The beads were then harvested. They were then washed several times with distilled water and finally with fresh medium. The beads were then distributed into aliquotes with fresh medium.

3.1.2.4 Media for isolation

Various media like DPM (Baker & O'Keefe, 1984), F (Simonet *et al.*, 1985) , OS-1 (Dobritsa & Stupar, 1989) and Q_{mod} (Lalonde & Calvert, 1979) were tried for isolating *Frankia* (for the compositions of media see appendix II).

3.2 PLANT INFECTION TESTS

3.2.1 Seed germination and seedling cultivation

Seeds were surface sterilized with 30% H_2O_2 for 10 minutes and rinsed several times with sterile distilled water. The seeds

of *Alnus nepalensis* were placed on a sterile moist filter paper and kept in the BOD incubator at 26±3 °C for germination. Since the *Casuarina* seeds float, they were allowed to germinate on the surface of sterile water in a jar at 30±3 °C in an incubator.

Fifteen days old seedlings were transferred to sterile pouches containing different concentrations of Hoagland solutions (1/4, 1/8 & 1/16) without Nitrogen (for composition and see Appendix II). The following three sets were prepared for each isolate:

1. Seedlings inoculated with crushed nodule suspension (+ve control) (Ref. 3.1.2.4 for the preparation of nodule suspension).
2. Seedlings inoculated with 100µL of 30 day old *Frankia* pure culture under test.
3. Uninoculated seedlings (-ve control).

Twenty seedlings were used in each set. The *Alnus* seedlings were allowed to grow in a plant growth chamber at 26°C with approximately 90% relative humidity and 11000 lux illumination.

3.3 NITROGENASE ACTIVITY

Acetylene reduction assay (ARA) (Burris, 1974) was employed to measure the nitrogenase activity of the isolates. Three mL of 30 day old subculture of each isolate, were taken in 9mL stoppered vials. One mL of air was replaced with 1 mL of acetylene. The vials were incubated at $28\pm1^{o}C$ and were shaken time to time. Ethylene production from acetylene was measured with a "Tracor 540" gas chromatograph fitted with "Porapak-T" column. The chromatograph used hydrogen as fuel and nitrogen served as carrier gas.

3.4 POST ISOLATION WORK UP

3.4.1 Maintenance of culture

After successful isolation of *Frankia* in pure culture, the isolates were maintained in two different media (Q_{mod} & DPM). Since different strains of *Frankia* preferred different carbon sources, the DPM medium was modified. in mod DPM, more than one carbon source were used (for the composition of modDPM see table 3.1).

TABLE 3.1

Composition of culture medium

modDPM

Constituents	Concentrated stock solution (per liter)	Working solution
(A) MACRONUTRIENTS	10X	
KH_2PO_4[1]	10.0 g	7348 μM
$MgSO_4$[2]	1.0 g	0899 μM
$CaCl_2.2H_2O$[2]	0.1 g	0068 μM
Sodium propionate[4]	12.0 g	12490 μM
Glucose[3]	100.0 g	55506 μM
Sodium acetate[3]	12.0 g	8780 μM
Tween80[3]	10 mL	
(B) MICRONUTRIENTS	1000X	
H_3BO_3[3]	2.860 g	46.3 μM
$MnCl_2.4H_2O$[2]	1.810 g	9.1 μM
$ZnSO_4.7H_2O$[2]	0.220 g	0.8 μM
$CuSO_4.5H_2O$[2]	0.080 g	0.3 μM
Na_2MoO_4[2]	0.025 g	0.1 μM
$CoCl_2.7H_2O$[2]	0.025 g	0.1 μM
(C) IRON	1000X	
$FeSO_4.7H_2O$[1]	10.008 g	370.4 μM
Na_2 EDTA[3]	13.410 g	36.0 μM

pH—7.00

[1]HiMedia laboratories, Bombay, India
[2]E Merck (India) ltd. Bombay, India
[3]Qualigens fine chemicals Bombay, India
[4]Sigma chemical co. St. Louis, USA

3.4.2 Decontamination

To recover the cultures which were contaminated by other microorganisms, different antimicrobial agents were used. For instance, 500 µg/mL of Cycloheximide (Actidione[TM], for structural formula see Appendix IV) were used to eliminate fungal contamination (Actidione inhibits protein biosynthesis in eucaryotic cells by binding 80S ribosome). Since *Frankia* is a gram+ve bacteria, 10µg/mL of Nalidixic acid (1-Ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid) (Sigma chemical Co. USA, for structural formula see Appendix IV) was used. For gram+ve bacteria, N_2 free medium like DPM was used and subsequently plating was done.

3.5 STRUCTURE AND ULTRASTRUCTURE

3.5.1 Light microscopy

Root nodules, which were collected from different parts of India (see Chapter 3.1.1), were fixed in FAA (90mL of 70% ethanol, 5mL glacial acetic acid and 5mL formalene). They were

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[TM]Actidione is the registered trade mark of Upjohn Co. USA.

then dehydrated in a series of extra pure acetone (S D fine chem Ltd. Buisar, India) and embedded in paraffin wax. Longitudinal and transverse sections, 6-10 μm thick were cut with a microtom (Leitz 1512, Germany). The safranin-fast green staining procedure (Sass, 1958) was used to stain the cortical and xylem cells differentially in order to study their relative arrangement. The endophyte in the nodule was stained using the Brown & Brenn (1931) staining procedure.

3.5.2 Scanning electron microscopy of *Frankia in vivo* & *in vitro*

Scanning electron microscopy was done both with pure cultures as well as *Frankia* within the nodules.

3.5.2.1 Fixation, washing & post fixing

For Frankia *in pure culture:*

One month old *Frankia* culture was taken in a microcentrifuge tube and the cells were allowed to settle down. Upper aqueous phase containing the medium was decanted carefully. One mL of 3% glutaraldehyde was added and cells were allowed to fix for 4 h.

Glutaraldehyde was decanted carefully and the cells were struck on glass stubs with the help of egg albumin Then the cells were washed thoroughly in 0.1 M cacodylate buffer and post fixed for 2 h in 1% OsO_4.

For Frankia *in side the nodules:*

Each nodule lobe was cut separately and washed thoroughly with distilled water. The nodule lobes were then fixed for 4 h in 3% glutaraldehyde and washed thoroughly with 0.1 M cacodylate buffer. They were then post fixed for 2 h. in 1% OsO_4.

3.5.3.2 Dehydration

In both cases, dehydration was done with a series of acetone (30%, 50%, 70%, 80%, 90% & 95%), keeping 30 min. in each grade with two changes followed by one hour in dry acetone with two changes. However, the nodules were kept for 24 h in each step for better dehydration.

3.5.2.3 Sectioning of nodules

The dehydrated nodules were then embedded in paraffin wax Transverse sections (10 μm) thick were cut with a microtome

(Leitz 1512, Germany). The sections were mounted on glass stubs and the wax was removed with xylene.

3.5.2.4 Critical point drying

Critical point freeze drying of the dehydrated *Frankia* cells was done using liquid CO_2 with a Polaron E3000 apparatus. The drying was carried out at 31.5^{o}C at 1100 p.s.t.

3.5.2.5 Gold sputtering

Gold coating of *Frankia* cells as well as nodule sections was carried out with a JEOL Fine Coat JFC-1100 ion sputter under vacuum in an inert atmosphere. Coating of uniform thickness was obtained by evaporating gold on the specimen kept at specific distance and at specific time.

The specimen were then fixed on metal tablets with Dotite-Electroconductives and observed under scanning electron microscope (JEOL JSM-35cF).

3.5.3 Transmission electron microscopy of nodule and pure culture

3.5.3.1 Collection of material

Root nodules collected from different parts of India, were thoroughly cleaned with water and detergent solution. The epidermis was peeled off, nodules were cut into small pieces and then kept for fixation.

For pure culture, liquid cultures were taken in microcentrifuge tubes and centrifuged at 5000 rpm for 5 minutes. The pellet was saved and washed twice with distilled water. Then few drops of 2% molten agarose were poured in the tubes. After the agarose solidified, the blocks were taken out by cutting the tube. These were cut into suitable pieces and kept for fixation.

3.5.3.2 Fixation

The nodule lobes as well as agarose embedded pure cultures of *Frankia* were fixed in glutaraldehyde (3%) in 0.3M cacodylate buffer (for composition and preparation see Appendix III) at 27°C and 4°C for 4 h each.

Fixatives were prepared with buffer solutions and the fixation was carried out at pH 7.2 to 7.4.

3.5.3.3 Washing

Following fixation in the primary fixative the tissues were washed in 0.1M cacodylate buffer and kept over night in the same buffer.

3.5.3.4 Post fixation

The tissues were post fixed in OsO_4 for 2 hours at 4^oC and washed thoroughly with cacodylate buffer for 20 minutes.

3.5.3.5 Dehydration

The tissues contained ~95% water which needed to be removed. For that tissues were passed through a dehydration series of acetone (30, 50, 70, 80, 90, 95, 100% acetone respectively) twice for 15 minutes each followed by 1 hour in dry acetone with two changes.

3.5.3.6 Cleaning off the acetone

To facilitate better infiltration tissues were cleaned off with Epoxy propane.

3.5.3.7 Infiltration and embedding

The tissues were then kept in propylene oxide for 25 minutes. They were then embedded in epon mixture (1:1 mixture of propylene oxide: epon, for one hour: subsequently for 24 hours in a 1.4 mixture and finally in 100% epon for four days). Resin capsules were then gradually polymerized by keeping them at 35°C, 48°C and 60°C at 24 h each respectively.

3.5.3.8 Ultrathin section for TEM

To achieve a high resolution from the Electron Microscope, the section of the specimen had to be less than 600 A (60 nm) thick. Such thin sections were obtained on ultramicrotome (LKB-BROMMA 2088 ULTROTOME^{TM} V).

3.5.3.8.1 Trimming of blocks to pyramidal cones

This was done with a simple blade. While trimming the block care was taken to make the cutting face of the specimen block as small as practicable by trimming away all the free resin.

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^{TM}Ultratom is a trade mark of Pharmacia fine chem., Uppsala, Sweden.

3.5.3.8.2 Knives

Glass knives were used. The knives were made out of good glass-strips (Belgian) with the help of LKB-BROMMA 7800 KNIFEMAKER. The sections cut on the ultramicrotome needed to be floated in pool of distilled water and for that purpose, plastic trough was used. It was fitted up to the knife edge.

3.5.3.8.3 Preparation of semithin sections for optical microscopy

To determine the state of the embedded specimen, the quality of the fixation, the area of the ultrathin section, the size and position of the cutting face for final trimming etc, it is necessary to prepare 0.5 to 2.0 μm thick sections.

The semithin sections floating in water were lifted with a thin brush and placed in a clean glass slide. The slides were placed on a hot plate at about 80^{o}C and dried. Toludiene blue was used to stain the sections.

3.5.3.8.4 Preparation of ultrathin sections

After scanning the sections under the optical microscope, the area to be examined under TEM was selected and blocks were further trimmed. Fresh glass knives were used for cutting

ultrathin sections. On the liquid in the trough, the ultrathin sections showed different colours. Only grey sections were considered because the grey sections were ~60 nm thick followed by silver(~60-90 nm) and gold (~90-150 nm).

The ultrathin sections on the water were lifted on specially made metal grids made of copper (Agar scientific Inc. USA).

3.5.3.8.5 Staining ultrathin sections

To obtain a good contrast, a double staining method using uranyl acetate (Uranyl acetate Dihydrate, $UO_2(OCOCH_3)_2.2H_2O$, M_r424.15, Fluka Chemika-BioChemika, Switzerland) and lead citrate (Citric acid Lead salt, $(C_6H_5O_7)2Pb_3.3H_2O$, M_r1053.83, Fluka Chemika-BioChemika, Switzerland) was followed for histological studies.

Staining procedure

The strains were filtered with 0.22 µm syringe membrane filter. The grids carrying the sections were placed on a watch glass and few ml of uranyle acetate was poured. The whole watch

glass was covered with a card-board cover because the staining is effective when carried out in dark. After staining for 10-15 min. each grid was washed in two lots of 50% ethanol and 2 lots of double distilled water with continuous agitation and dried carefully on a filter paper keeping the surface of the grid not carrying the sections on the filter paper.

Few mL of lead citrate was placed in a watch glass and the grids were placed on it and kept for 10 min. Then the grids were washed with 0.1 N sodium hydroxide and then in 2 lots of double distilled water. The grids were dried and stored in a petridish.

The sections were then observed in transmission electron microscope (JEOL JEM-100 CXII).

3.6 DEOXYRIBONUCLEIC ACID BASE COMPOSITION OF THE DNA OF *FRANKIA* ISOLATES

3.6.1 The isolates

Four isolates of *Frankia* were used in this study. The isolates were ACN^{1AG}, CeAR16, CeAR44, and CeRS2. All the cultures

were grown in Q_{mod} medium (Lalonde and Calvert, 1979). About 4mL of stock cultures were transferred to a 200 mL Erlenmeyer flask containing 100 mL medium. The cultures were incubated in the dark at 28±3°C without agitation and were allowed to grow for three weeks. The cells were harvested by centrifugation and stored at -20°C for further use.

3.6.2 Preparation of nucleosides from *Frankia* genomic DNA.

The nucleosides of *Frankia* genomic DNA were prepared in the following manner:

1. Ten mL of one month old *Frankia* culture was washed with 10 mL TE-8 buffer (for composition see Appendix III) and suspended in 10 mL of the same.
2. The cells were sonicated at 23 kHz for two minutes and then 1mg of Proteinase K (Sigma chemical co., St. Louis, USA) was added. Tubes were incubated for 1h at 37°C. SDS was added to arrive at a final concentration of 1%, and tubes were reincubated at 60°C for 10 min.
3. The solution was then distributed into two aliquots and equal

volume of molecular biology grade phenol was added. Two phases were mixed gently.

4. These were then centrifuged at 5000 rpm and took the upper aqueous phase was taken in a new tube (interphase was avoided).

5. Equal volume of chloroform was then added, tubes were inverted gently to ensure proper mixing and were centrifuged at 5000 rpm for 5 minutes at room temperature.

6. The upper aqueous phase was taken in separate tubes. Equal volume of iso-propyl alcohol was added to each tube, mixed gently and kept overnight at room temperature.

7. The solution was then centrifuged at 13000 rpm for 30 minutes at 20^{o}C.

8. The DNA pellets were saved and dried in vacuum desiccator. The DNA was resuspended in 100 µL of ultra pure water.

9. 50 µg/mL RNAase along with 10X RNAase buffer (0.2M Tris.HCl, pH-8.0) were added to the tube and the tubes were incubated for 30 min at 37^{o}C.

10. Steps 3-8 were rep ed again.

11. To 10mL of DNA solution thus obtained, 20 µL of 5mM sodium phosphate buffer (pH-7.1) in 0.5Mm $MgCl_2$ and 10 µL of enzyme soln.A (10µL contains 2µg Pancreatic DNAase, 10486g Venom Phosphodiesterase, 10µg RNAase A and 2.5µg Bacterial alkaline phosphatase, all from Sigma chemical co., St. Louis, USA) were added. It was incubated for 18h at 37°C.

3.6.3 Direct nucleoside analysis by fast protein liquid chromatography (FPLC™).

Purified nucleosides were injected directly to a PepRPC™ column fitted to a Pharmacia FPLC. Nucleosides were eluted out from the column with 40mM/L ammonium acetate buffer (pH-5.0). ACN^{1AG} was used as reference strain.

3.7 ISOLATION OF DNA

3.7.1 Isolation of total genomic DNA from *Frankia* pure culture

Total genomic DNA was isolated following the protocol described below.

™FPLC and PepRPC are the registered trade marks of Pharmacia fine chem., Uppsala, Sweden.

centrifuged at 2000 rpm and supernatent was discarded.

2. The pellet was resuspended in 1 mL of TE 8 buffer (see Appendix III) and transferred to a 'Tarsons' microcentrifuge tube.

3. It was centrifuged at 7000 rpm for 15 min and pellet was dissolved in 1 mL of TE 8 buffer.

4. The solution was forced through 1 mL sterile syringe needle to break the colonies.

5. 10 mg/mL of molecular biology grade lysozyme (Sigma chemical Co., St. Louis, USA) and a pinch of achromopeptidase (Sigma chemical Co., St. Louis, USA) were added to the solution and it was incubated at 20^{o}C for 60 min.

6. 250 µL of 20% SDS (20 g SDS in 900 mL of water) was added. It was Kept at 60^{o}C for 30 min and at room temperature for 15 minutes.

7. The solution was then divided equally into two tubes (625 µL/tube).

8. To each tube 625 µL of molecular biology grade phenol

(Bangalore Genei Ltd., Bangalore, India) was added. Then the two layers were mixed gently without vigorous shaking.

9. The tubes were centrifuged at 5000 rpm and the upper aqueous phase was taken in a new tube.

10. Equal volume of chloroform (600 µL) was added gently and centrifuged at 5000 rpm for 5 min.

11. The aqueous phase was taken in a separate tube. Iso-propyl alcohol (360 µL) was added and the tube kept overnight at room temperature.

12. Next day, the tubes were centrifuged at 20°C for 30 min at 14000 rpm.

13. The liquid phase was decanted and the pellet was saved and dried with a vacuum desiccator and the DNA was resuspended in 10 µL of ultrapure water.

3.7.2 Isolation of DNA directly from nodules

1. Nodule lobes were sterilized with 30% (V/V) H_2O_2 for 15 minutes.

2. One lobe from each nodule was taken and peeled.

3. Each lobe was crushed with a plastic pestle in 300µL of extraction buffer (for composition and preparation see Appendix III) in 1.5mL microcentrifuge tube.

4. The homogenate was incubated at 65°C for 1 hour and then centrifuged at 7000 rpm for 10 minutes. The supernatent was saved.

5. This supernatent was extracted with an equal volume of chloroform:isoamyl alcohol (24:1)(V:V), and centrifuged at 13000 rpm for 30 minutes. The aqueous phase was taken in a separate tube and DNA was precipitated with 2 volumes of cold absolute ethanol and centrifuged at 13000 rpm at 4°C for 30 minutes. The DNA pellet was washed with 70% ethanol, vacuum dried and resuspended in 10µL of ultrapure water.

3.8 ANALYSIS OF DNA IN AGAROSE GEL

3.8.1 The agarose gel electrophoresis of DNA

The average size of the DNA molecule was analyzed using the protocol mentioned below:

1. Molecular biology grade, DNAase free 0.8% agarose (gelling temperature 36°C) (product No. A 9539 Sigma chemical co., St. Louis, USA) was dissolved in 30 mL of 0.5X TBE buffer (for composition and preparation see Appendix III) by keeping on a heating mantel.

2. The gel was cast on a gel platform (100 X 70 mm). An eight-well comb was used to make wells.

3. After solidification, the gel platform along with the gel was put into a submarine electrophoresis chamber keeping the well-end towards the cathode side.

4. Two µL of DNA soln. was mixed with 5 µL of loading buffer (Type III, Sambrook *et al.*,1989) (for composition and preparation see Appendix III) and directly loaded into the wells after submerging the gel in TBE buffer in the tank.

5. Four volt/cm current was applied with a Sigma PS250 (Sigma chemical co., St. Louis, USA) power supply.

6. After 2.5 h, the gel was taken out of the tank and stained with 0.5 µg/mL ethidium bromide (2,7-diamino-10-ethyl-9-

phenylphenanthridinium bromide, $C_{21}H_{20}BrN_3$, M_r 394.33, Sigma chemical co., St. Louis, USA) (for structure see appendix IV) for 30 minutes at room temperature.

7. Destaining was done by soaking the stained gel in water for 20 min. at room temperature.

8. The gel was then viewed on a transilluminator (Pharmacia fine chem, Uppsala, Sweden).

3.8.2 The gel photography

Photographs were taken with a 35 mm camera (Pentax K_{1000}) using an orange filter keeping the gel on a transilluminator.

3.9 PURIFICATION OF DNA

The DNA isolated by the procedure described above was contaminated with phenol, chloroform, alcohol etc. which reduced the efficiencies of restriction enzymes. To overcome these difficulties, DNA was purified by the following protocol.

1. Two and half vol. of NaI soln. (saturated at 30°C) was added to the DNA soln.

2. Then 8 µL of glass milk (Bangalore Genei ltd., Bangalore) was added to the mixture.

3. The contents were mixed thoroughly and left at room temperature for 5 min with occasional mixing. That allowed adsorption of DNA molecules to the glass solution.

4. It was then centrifuged at 12000 rpm for 5 sec and pellet was saved.

5. The pellet was washed with wash buffer (200 µL) (for composition see Appendix III) and centrifuged at 12000 rpm for 5 second. The supernatent was discarded.

6. The steps 5-6 were repeated twice.

7. The DNA was eluted adding 20 µL of 1 X TE buffer (for composition and preparation see Appendix III) to the pellet. After resuspending the pellet, the soln. was incubated at 50^{o}C for 5 min.

8. It was then centrifuged at 12000 rpm for 15 sec and supernatent was collected in a separate tube.

9. Steps 8-9 ware followed twice.

10. The efficiency of the elution was checked on agarose gel as described in 3.8.

3.10 PCR AMPLIFICATION OF *FRANKIA* DNA BOTH FROM PURE CULTURE AS WELL AS FROM THE NODULES

3.10.1 PCR mixing

Since there was a chance of contamination of aerosol DNA, the mixing was done in a UV sterilized protected chamber. The custom oligonucleotide primers were prepared by ordering from Bangalore Genei pvt. ltd. Amplification of *rrn* region was performed using primers MGL4 (5′-GGGGTCCGTAAGGGTC-3′16S coordinates 989 ac) (Bosco *et al.*, 1992) and MGL7 (5′-AAGGAGGGGATCCAGCCGCA-3′ 16S 1490-1509′). The nif region was amplified using the primers MGL1 (5′-CACTGCTACCGGTCGATGAA-3′ *nif*D coordinates 807) and MGL2 (5′-CGAGGTAGGTCTCGAAACCGG-3′ *nif*K coordinates 700) (Jamman *et al.*, 1993). Lyophilized primers were dissolved in ultrapure water to make a 1mM stock solution. This was further diluted into 5μM working solution and was used at a final concentration of 0.1μL/μL (V/V).

Each GeneAmp™ dNTP (part # N808-0007) was used at a final concentration of 0.2ng/μL.

AmpliTaq™ DNA polymerase was used at a final concentration of 0.05U/μL.*

Geneamp™ 10 X PCR buffer supplied with AmpliTaq™ DNA polymerase was used at a final concentration of 1X (for composition see Appendix III).

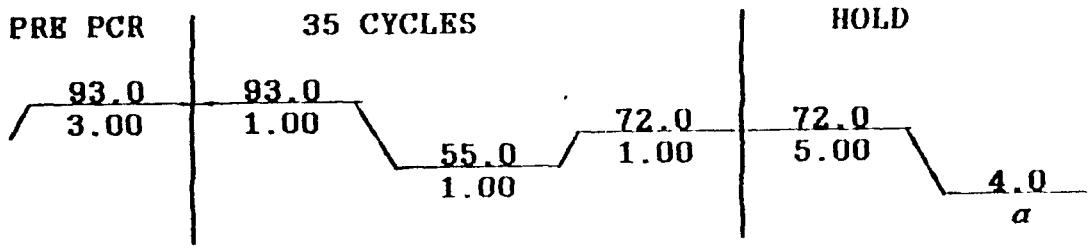
DNA isolated from nodules as well as from pure cultures (see 3.6) was diluted 100 times and was used at a final concentration of 0.04μL/μL (V/V). Total volume was made up with ultrapure water.

Reaction volume per MicroAmp™ tube was usually 50μL. They were placed in a GeneAmp™ PCR system 2400 and ran using the following programme:

---

*Unit Definition: One unit of the enzyme was defined by the suppliers as the amount that will incorporate 10 n mols of dNTPs into acid insoluble materials per 30 minutes in a 10 minute incubation at 74°C.

™MicroAmp is a registered trade mark of Perkin Elmer Incorporation, USA. While, AmpliTaq and GeneAmp are registered trademarks of Roche Molecular Systems, Inc. USA.



To check the efficiency of amplification, 1/10 of the PCR products were visualized by electrophoresis on 0.5μg/μL ethidium bromide containing 0.8% & 2.0% agarose gel (for *nif* and *rrn* regions respectively) at 30 V/cm for 2 hours.

3.11 RESTRICTION DIGESTION AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM OF PCR AMPLIFIED DNA FRAGMENT

3.11.1 Restriction Digestion

1. Seventeen μL of PCR amplified *rrn* region as described in 3.10 was taken in a sterile microcentrifuge tube.
2. Two uL of 10X restriction enzyme digestion buffer (supplied with restriction enzyme by the manufacturers, for composition see Appendix III) was mixed.
3. Restriction enzyme *Nci*1, (obtained from *Neisseria cinerea*, Recognition site $CC\downarrow{}^{C}_{G}GG$, New England Biolab, USA) was

quickly added (20 units*). The soln. was mixed thoroughly.

4. The tube was placed on a float rack and incubated in a water bath at 37°C for 2 hours.

5. The reaction was stopped by adding 0.5M EDTA (pH 8.0) to a final concentration of 10 mM.

6. The digested DNA was kept at -20°C until further use.

3.11.2 Analysis of RFLP

1. For RFLP analysis molecular biology grade agarose (Sigma chemical co., St. Louis, USA) was dissolved in 60 mL of TBE buffer at a final concentration of 4% (W/V).

2. Ethidium bromide soln. (Sigma chemical co., St. Louis, USA) was added to the soln. at a final concentration of 1 µg/mL.

3. The gel was cast on a gel platform (130 X 65 mm) putting a comb.

---

*__Unit Definition:__ One unit is defined by the suppliers as the amount of enzyme required to digest 1µg lamda DNA in 1 hour at 37°C in 50µL assay buffer.

4. The DNA was loaded with 5 uL of loading buffer.

How much vol ?

5. The electrophoresis was carried out with low voltage (0.13 volt/cm) for 6 h.

6. The gel was visualized on Transilluminator (Pharmacia, Sweden) and photographed using Leitz macrophotography system and Ilford FP4 plate films as well as Pentax K_{1000} camera and 35mm ORWO 35mm B/W film.

Chapter 4

RESULTS AND DISCUSSION

4.1 COLLECTION OF GERMPLASAM

The abundance of nodules varied from place to place (Table 4.1). Frequency of nodulation was more in the plants found in coastal areas. Surprisingly, in Rajasthan desert's sandy soils, I could not detect any nodules even upto a depth of half a meter. Clearly it is the proximity to the sea that induces more nodulation in coastal areas. This may be because of water stress that trees of Rajasthan must have faced. In costal areas, available nitrogen tends to leach out while lack of soil humus in sandy soils would reduce levels of available nitrogen. Both these situations were conducive to profuse nodulation. The size of the clumps of nodule lobes varied from 1.5 cm to 15 cm (Plate B-1).

Nodules were located in the soil at depths ranging between 3cm to 500cm and were found mainly on the developing lateral roots. Few nodules were found in the crown area.

Actinorhizal nodules are perennial and generally comprise of numerous conical shaped lobes (plate B-2). In the case of most

Plate B

1. Nodule clump from *C.equisetifolia.*

2. Close up view of a typical *Myrica* type nodule lobe from *C. equisetifolia.*

3. Scanning electron micrograph of surface of *Casuarina* nodule showing population of rhizospheric microorganisms.

4. Scanning electron micrograph showing both infected (curled, inoculated with CeRS2) and healthy root hairs of *Casuarina* seedlings.

5. Scanning electron micrograph of infected *Casuarina* root hair showing the hook shaped structure.

6. Scanning electron micrograph of *Alnus nepalensis* root nodules showing root hair branching.

7-10. Stages of nodule development.

15KU X400 1650 10.0U RSICS
15KU X540 1657 10.0U RSICS
9486 10.0U RSICS

Plate B

TABLE 4.1.

Percent nodulation and morphological features of *Frankia in vivo* in two actinorhizal plants collected from various sites

Plant	Name of the place	%of nodulation*	hyphae	vesicles	sporangia
Casuarina equisetifolia	Bhubaneswar	65	+	-	-
	Digha	92	+	-	-
	Guwahati	25	+	-	-
	Hyderabad	76	+	-	+
	Itanagar	81	+	-	-
	Madras	80	+	-	-
	Puri	68	+	-	-
	Rameswaram	100	+	-	-
	Siliguri	66	+	-	-
	Thiruchirapalli	50	+	-	-
	Trivandram	64	+	-	-
Alnus nepalensis	Arunachal	48	+	+	-
	Darjeeling	71	+	+	-
	Mawlai	59	+	+	-
	Upper Shillong	52	+	+	-

*See text for details. +=present, -=absent.

actinorhizal nodules, a proliferation of cortical cells near the infected deformed root hair leads to the differentiation of modified lateral roots, which become the lobes of the nodules. Thus, each of the lobes is a diminished lateral root.

Developing nodules were whitish yellow in colour and turned brownish later. Zhang and Torrey (1985) have grouped actinorhizal nodules in to two types, the *Alnus* type and the *Myrica* type. *Alnus, Elaeagnus* etc. represent the first type while, *Casuarina, Myrica* etc. represent the second type. All the nodules collected under the present study were of *Myrica* type.

In case of *Casuarina*, nodule bears nodule roots which arise from the distal region of each mature nodule lobe. Immature nodules lack nodule roots but sometimes have a cap like structure at the distal end.

4.2 ISOLATION OF ENDOPHYTE

4.2.1 Surface sterilization

Since many soil organisms are found at the outer epidermal layer of actinorhizal root nodules (Plate B-3), elimination of

such contaminants is a prerequisite for successful isolation of *Frankia*. Several methods were tried for surface sterilization (see 3.1.2.1). The technique using H_2O_2 as sterilant followed by the removal of outer epidermal layer and chopping of nodule lobes gave best results. *Frankia* is a sluggish microorganism and only a few slices produced *Frankia* colonies (Plate C-1). Each colony originated from one unit that has been called as positive cells by (Diem and Dommergues, 1983). Now each nodule slice bears a very limited number of these units. Among the positive units those that come in contact with the nutrient medium produce colonies. These are called UFF (units able to form *Frankia*) (Diem *et al.*, 1982). The term UFF was defined broadly to refer either to specific structures or simply to hyphae or clusters of hyphae able to grow out of the nodule. The fact that new growth of *Frankia* often originated from clusters of hyphae was mentioned by Berry and Torrey (1979), which suggests that clusters of hyphae (preferably in the form of nodule slices) may be more suitable for initiating colonies than finely fragmented structures (crushed nodules)

Plate C

Scanning electron micrograph of

1. A typical *Frankia* colony (CeRS2).

2. A part of the colony of CeRS2, showing hyphae(h), vesicle(v) and vesicle stalk (st).

3. A part of the colony of CeDI1, showing developing sporangia.

4. Mature sporangia(S) with spores(sp) of CeRS2.

5. A part of the CeDI1 colony showing vesicles.

6. CeDI1 showing intercalary globos sporangia like structure (single arrow) and short septate torulose hyphae (double arrow).

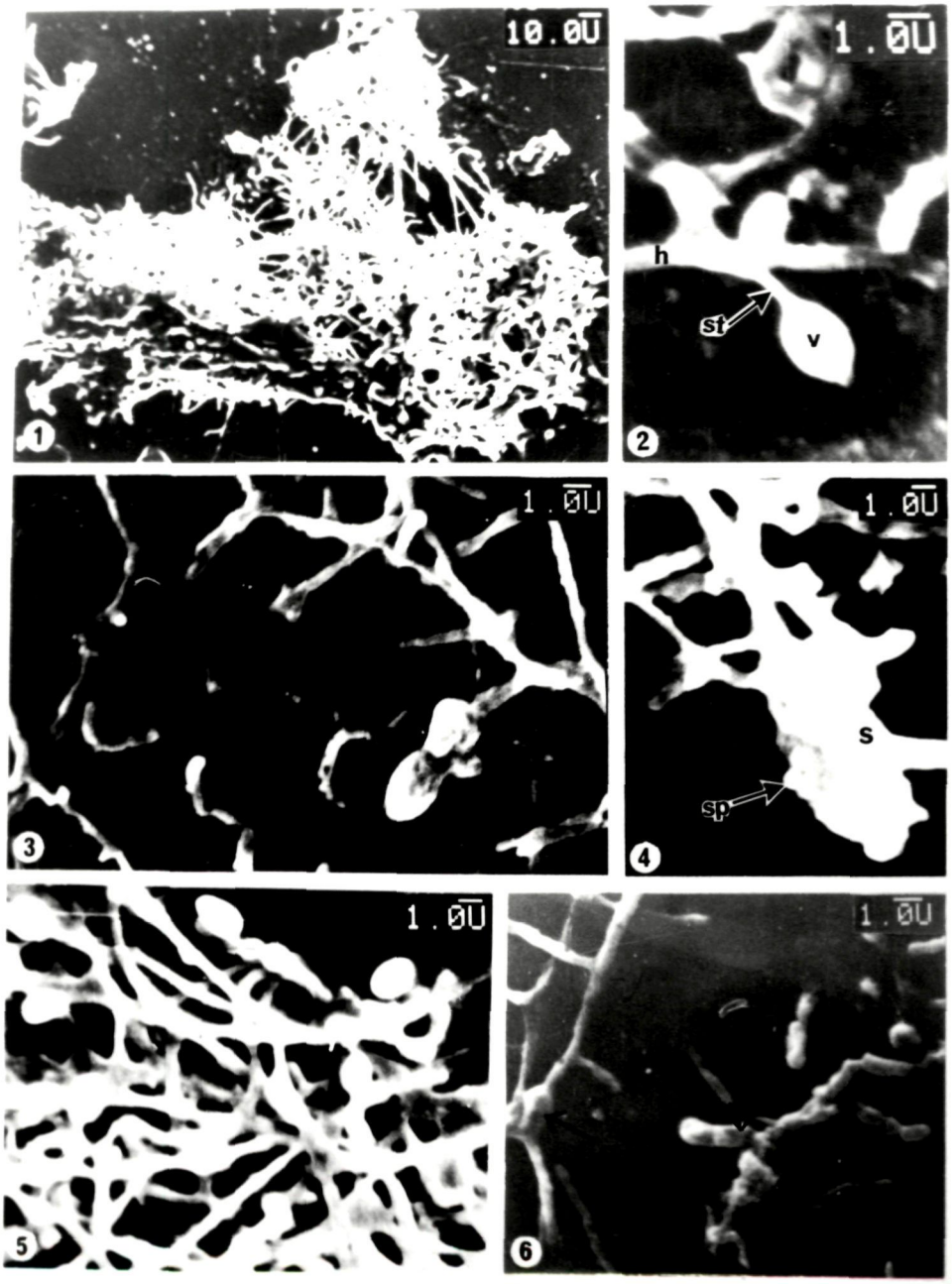


Plate C

Plate D

1. Scanning electron micrograph of a section of CeRS2 induced nodules showing *Frankia* hyphal mat inside the cortical cells.

2. Close up view of the same.

3. *Frankia* hyphae inside the cells of field collected *Casuarina* nodules.

4. Scanning electron micrograph of 15 day old air dried alginate bead.

Plate D >>>>>

4.5.2.2 Study of Frankia in vitro *and* in vivo

Scanning electron micrographs of pure culture of *Frankia* show hyphae, vesicles and sporangia.

4.5.2.2.1 Hyphae

The hyphae were branched and ranged in width from 0.65 to 0.70 μm (Plate C). When sections of the nodules, both induced as well as field collected, were examined, the mean diameter of the hyphae was found to be 0.53 and 0.55μm respectively (Fig. 4.2). The difference between the hyphal diameter of *in vitro* and *in vivo* conditions were significant at p=0.05 level. This may be due to the fact that the hyphae were very densely packed inside the host cell.

4.5.2.2.2 Vesicles

The vesicles are without doubt, the most definitive structures characterizing the genus (Plate C-2). Although functionally analogous in many ways to heterocysts of cyanobacteria, the vesicle is a unique developmental structure designed for physiological compartmentation and has not been described for any other

The colonies, as seen under light microscope, were cushion like and appeared to be embedded in mucilaginous material. Only hyphae located in the outermost periphery of the colonies could be stained with toluidine blue solution. The average colony was found to be about 300µm.

4.5.2 Scanning electron microscopy

4.5.2.1 Study of root hair infection

Root hair infection was studied after one week of inoculation. With light microscope it was seen that 50 to 60% of the root hairs were infected within one week. When studied under scanning electron microscope, typical root hair curling (Plate B-4), production of hook like structure (Plate B-5) and root hair branching (Plate B-6) were found. Unlike in *Elaeagnus*, in *Casuarina*, the microsymbiont enters through root hair. The path traversed by the endophyte probably causes the curling seen in Plate B-4.

like different carbon source, DPM was modified adding more than one carbon source (for composition see Table 3.1). This mod DPM resulted in better growth of isolates.

To overcome possible contamination during isolation and subculturing, cyclohexamide (actidion) and nalidixic acid were added to the culture medium. Growth of *Frankia* in the presence of these two additives was found to be normal.

4.5 MICROSCOPY OF FRANKIA

4.5.1 Light microscopy

Light microscopic studies of *Casuarina equisetifolia* root nodule sections demonstrated an organization different from other actinomycete-induced root nodules. The main difference was the absence of vesicles in the infected cells. Each nodule lobe consisted of an outer epidermis and a thick cylinder of cortex within which a single layer of endodermis surrounding the vascular cylinder or stele was present. Non infected cells were absent in the infected zone of the cortex. The infected cells were seen to be full of dense mat of hyphae.

reference to ACN^{1AG}. Burggraaf and Shipton (1983) have reported very low levels of acetylene reduction for strain AvcI1. Either their strain was a very poor fixer or their cultures were not pure enough. Specially since the study was conducted when not much was known about *Frankia*.

4.4 POST ISOLATION WORKUP

After the isolates were demonstrated to be *Frankia* both through microscopic observations as well as plant infection tests, the question arose as to what was the best medium for maintenance and growth of the organism isolated? Nitrogen sources like nitrate or ammonia surely promote the growth of *Frankia* isolates (personal observation). But I performed some experiments which needed cultures grown in nitrogen free media. For this reason, isolates were cultured in defined (nitrogen free DPM) as well as undefined (Q_{mod}) media. The growth rate of isolates in Q_{mod} was relatively higher than in nitrogen free DPM.

Regarding the carbon sources, isolates grew well with propionate (DPM) and glucose (Q_{mod}) but since different isolates

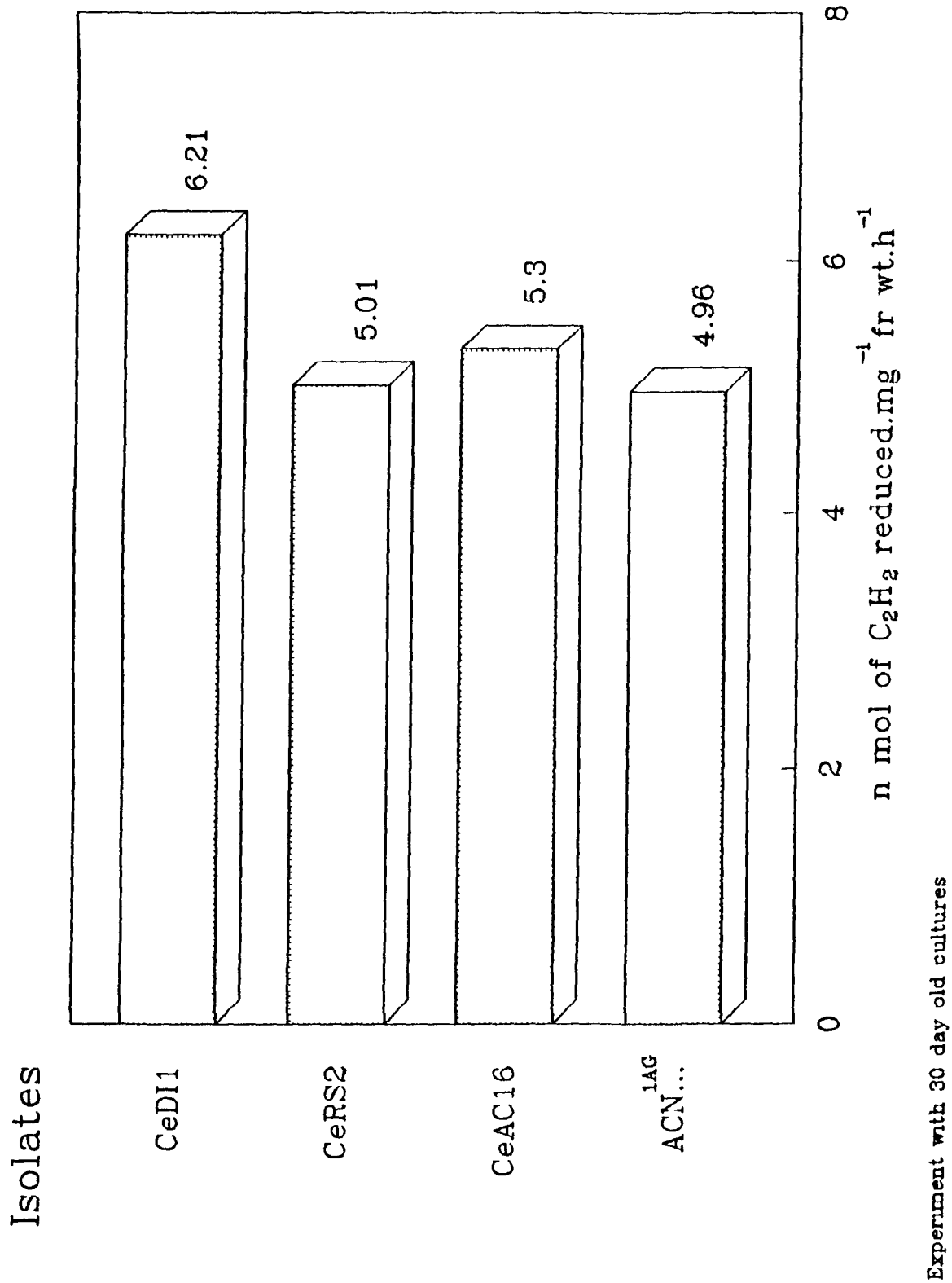


Figure 4.1 Nitrogenase activity for *Frankia* isolates.

nodules were seen (Plate B-7). There were 4-5 prenodules per seedling, but only 1-2 of them ultimately developed into complete nodules, which were visible five weeks after inoculation (Plate B-10). They were brownish in colour and 1-1.5 mm in diameter.

On the other hand, negative controls showed stunted growth in comparison to inoculated seedlings and at no stage of the experiment were nodules found in them. Chlorosis also occurred after certain period. These results confirm the ability of isolates to induce efficient nodules.

4.3 NITROGENASE ACTIVITY

Several isolates like CeDI1, CeRS2, CeAC16, CeAC44 along with ACN^{1AG} were tested on nitrogen free DPM medium for their nitrogen fixing capacity on 30th day. Results are compiled in Fig-4.1. These results clearly show the superiority of CeDI1 in terms of nitrogenase activity as demonestrated by acetylene reduction assay. The value is significant even at $p>0.01$ level while the activity of CeAC16 is significant at $P>0.05$ level with

TABLE 4.2

Frankia isolates obtained from nodules of actinorhizal plants collected from different parts of India

Isolates	Plant	Place
AnpM1	*Alnus nepalensis*	Mawlai, Shillong
CeAC16*	*Casuarina equisetifolia*	Arunachal Pradesh
CeAC42	*Casuarina equisetifolia*	Arunachal Pradesh
CeAC44*	*Casuarina equisetifolia*	Arunachal Pradesh
CeAC52	*Casuarina equisetifolia*	Arunachal Pradesh
CeAC413	*Casuarina equisetifolia*	Arunachal Pradesh
CeDI1*	*Casuarina equisetifolia*	Digha, W Bengal
CeRS2*	*Casuarina equisetifolia*	Rameswaram, T Nadu

*Isolates used for further studies

In the present study, successful isolation of *Frankia* from *Casuarina* root nodules was achieved by using Q_{mod} medium with lecithin. Table 4.2 lists the isolates obtained. Of these, only some were selected for farther studies.

4.2.4 Plant infection tests

Infection tests of *Casuarina equisetifolia* seedlings grown in pouches showed 55-60% of the plants formed 1-3 nodules within 3-4 weeks. Diem *et al.* (1983) reported 80% nodulation within 14-20 days. The less percentage and delayed nodulation in the present study may be because of the slow growth of *Casuarina* seedlings. *Casuarina equisetifolia* is a plant of chapparal and xeric ecosystems and predominant in the coastal dunes (Benson and Silvester, 1993). Probably that is the reason of *Casuarina* s sluggish behavior in Shillong atmosphere. Plants without young and developing root hairs hardly nodulate. This indicated that the presence of root hair is a prerequisite for nodulation.

Root hair deformation and curling took place within 1-3 days of inoculation (Plates B-4 to B-6). After nearly 2-3 weeks pre-

and fungi generally occurs through the presence of spores or hyphae. But in this technique along with inoculant, spores and hyphae are also trapped. So when they germinate a single bead gets contaminated. Constant vigilance and immediate elimination of contaminated beads would result in preventing contamination of the whole culture. Secondly, the time of first appearance of colonies under our conditions was about five days less compared to usual procedure.

To overcome the problem of mixed cultures, single spore colonies can be obtained by inducing artificial sporulation and subsequent entrapment of single spores in alginate beads.

4.2.3 Media for Isolation

Most media proposed for isolation of *Frankia* are supplemented with yeast extract and other diverse compound believed to promote *Frankia* growth e.g. vitamin B12 (Baker and Torrey, 1979), lecithin (Lalonde and Calvert, 1979) and alcoholic extract of root lipids (Quispel and Tak 1978).

According to Diem and Dommergues (1983), the amount of HFF in the nodule may be related to the age of the nodules, its physiological stage or host plant *Frankia* genomic determinates. I found that both the freshly collected young nodules as well as carefuly preserved nodules (at -20^{o}C) gave good results.

4.2.2 Isolation of *Frankia* in alginate beads

Distinct *Frankia* colonies appeared in alginate beads after 20 days of incubation as per the procedure described in 3.1.2.3. The most difficult part in the isolation of *Frankia* from root nodules was to get rid of contamination. It is well established that the growth of *Frankia* is very slow (generation time: 2-5 days, Diem and Dommergues, 1983), creating a high risk of over growth by contaminants (which may grow faster than *Frankia*) during the isolation procedure. Various workers have tried various methods to eliminate this problem. But, perhaps this is the first time that alginate beads were successfully employed to isolate *Frankia*. There are several advantages of this technique over the conventional ones. Firstly, contamination of bacteria

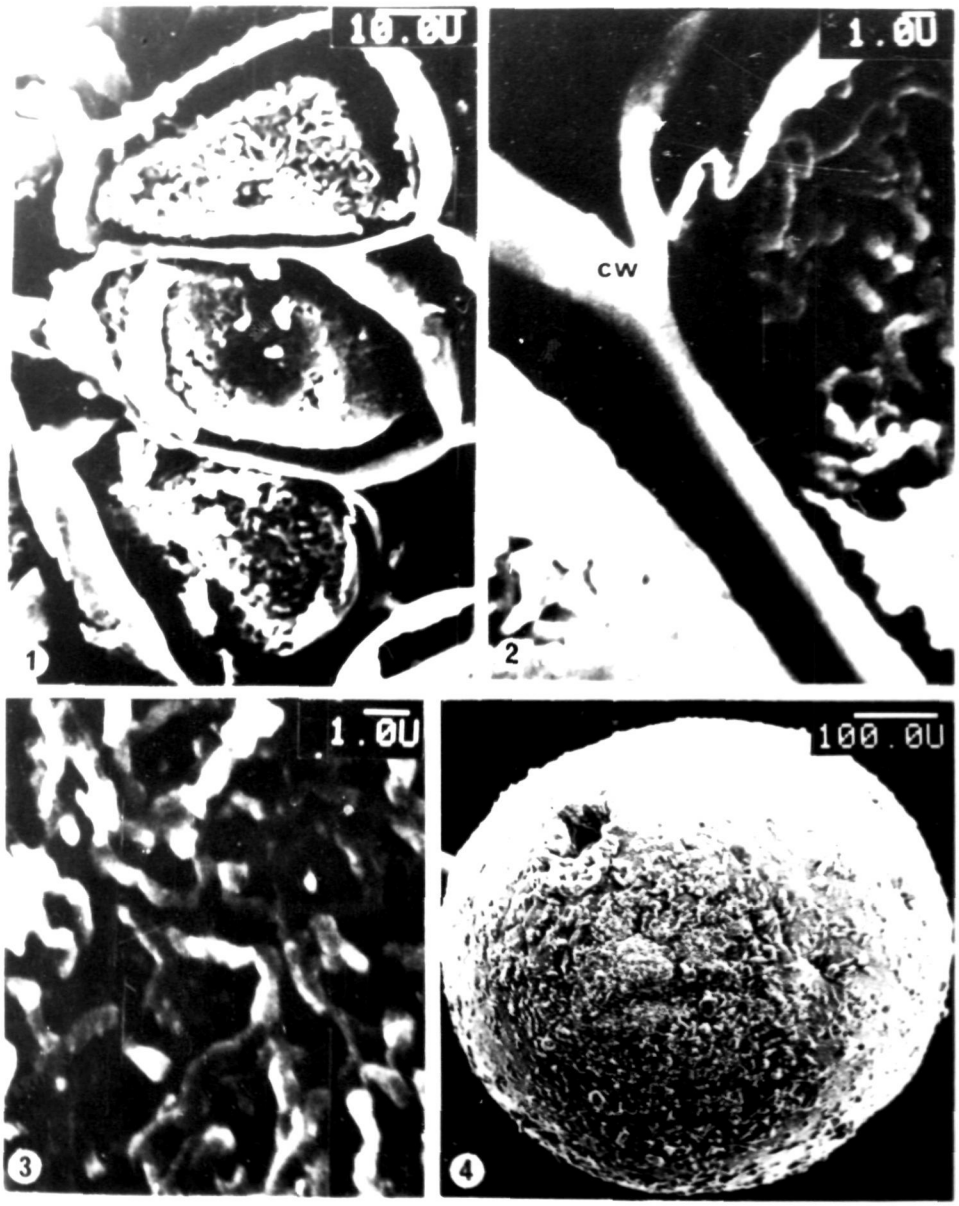


Plate D

Plate E

1. Transmission electron photomicrograph of the L S of field collected *Alnus nepalensis* nodule showing vesicle(v) and stalk(st).

2. Transmission electron photomicrograph of the L S of vesicle(v) of CeDI1 with stalk(st).

3. Transmission electron photomicrograph of *Alnus nepalensis* nodules showing T S of vesicles(v) surrounded by a void area from which vesicle envelop material has been extracted due to chemical fixation (arrow) (see text for details).

4. Transmission electron photomicrograph of T S of vesicle(v) in pure culture showing void area (arrow).

5. Transmission electron photomicrograph showing *Frankia* sporangia inside a *Casuarina* nodule. The spore contains the electron transparent reserve food material and the arrow indicates the outer electron dense layer followed by an electron transparent spore cell wall.

4.5.3.1 Hyphae

As mentioned earlier, hyphal diameter was found to be between 0.65 to 0.7 μm. TS of hyphae (Plate F-2) showed an outer cell wall and in the central region there was prominent electron translucent area called nucleoid (Newcomb and Wood, 1987). The nucleoid contains fibrillar material presumed to be DNA (Newcomb and Wood, 1987).

4.5.3.2 Vesicles

According to Benson and Silvester (1993), the plants provides an envelop outside both hyphae and vesicles. In vesicles, there is a distinct electron transparent area between the capsule and *Frankia* "cell wall", which has been called as a "void area" (Lalonde *et al.*, 1976). Newcomb and Wood (1987) had argued that this void area was present between the outer and the inner "cell wall". In my TEM studies of the TS of vesicles and hyphae too I observed clear "void area" (Plate E-1 to E-4 and F 1,2). With a view to ascertain the actual source of the void area, I did TEM studies of vesicles of cultured *Frankia* as well (Plate E-

that of typical *Frankia* spores (Plate C-4).

4.5.3 Transmission electron microscopy

Transmission electron microscopic observations of *Casuarina equisetifolia* and *Alnus nepalensis* root nodule sections showed that infected cells were totally occupied by the endophyte. In case of *Casuarina* no vesicles were found inside the infected cells. While in case of *Alnus* both hyphae and vesicles were present (Table 4.1). Berg (1983) also reported the absence of vesicles *in vitro* in *Frankia* of *Casuarina*, although, it did form vesicles *in vitro* (Zhang *et al*, 1984). An earlier report of morphologically distinct vesicles in *Casuarina* by Gardner (1976) has not been substantiated by numerous subsequent studies. May be the nodules collected by Gardner were not the nodules of *Casuarina* but of neighboring Actinorhizal plants of different genera. TEM studies were also done for pure cultures of *Frankia* isolates. Ultrastructure of different parts of *Frankia* both *in vitro* and *in vivo* is given below:

mented only in *Myrica* and *Alnus* sp. which also is sporadic and somewhat site specific (Torrey, 1987; Schwintzer, 1990). *In vivo* sporangia are rare in *Casuarina*. In this study nodules were collected from different parts of India but sporangia were found only from the nodules collected from Hyderabad. However, possibility of sp^+ nodules elsewhere can not be ruled out.

Another interesting feature is the presence of torulose hyphae after growth for one month in Qmod liquid medium. CeDI1 constituted short and wide hyphae with many septation and they principally evolved into intercalary oblong sporangia like structures (SLS, Plate C-6). SLS probably resulted from the enlargement of the hyphae followed by the formation of transverse or oblique septa. When mature, SLS were globose or subglobose in certain areas (Plate C-6); most were arranged in elongated torulose chains of cells. In contrast with the sporangia of *Frankia* from *Casuarina* described earlier (Plate C-4). SLS did not breakout easily but disrupted only under pressure into single unicellular spore like units, the shape and size of which differed from

prokaryote. Vesicles are borne either in the terminal or intercalary position of the hyphae. In the present study, only intercalary vesicles were seen. In all cases vesicles were pear shaped. The mean length of the vesicles in different isolates ranged from 2.47 to 2.07μm and diameter ranged from 1.33 to 1.47μm (Fig. 4.3). Small stalks which connected the vesicles with the hyphae were also present. The mean length and diameter of the stalk ranged from 0.97 to 1.33μm and 0.33 to 0.46μm respectively (Fig. 4.4).

4.5.2.2.3 Sporangia and sporangia like structures

Frankia usually produces multilocular sporangia which are unique to the *Frankia* group (Plate C-4). Though sporangia are produced readily in culture by *Frankia* isolates, in the present study out of the 4 isolates, sporangia were seen only in two cases. The length and diameter of the sporangia varied from 9.1 to 10.3μm and 3.86 to 4.28μm respectively (Plate C-4, Fig. 4.5). In symbiosis nodules may be sp^+ and sp^- apparently independent of their performance in culture. Sporangia in nodule is well docu-

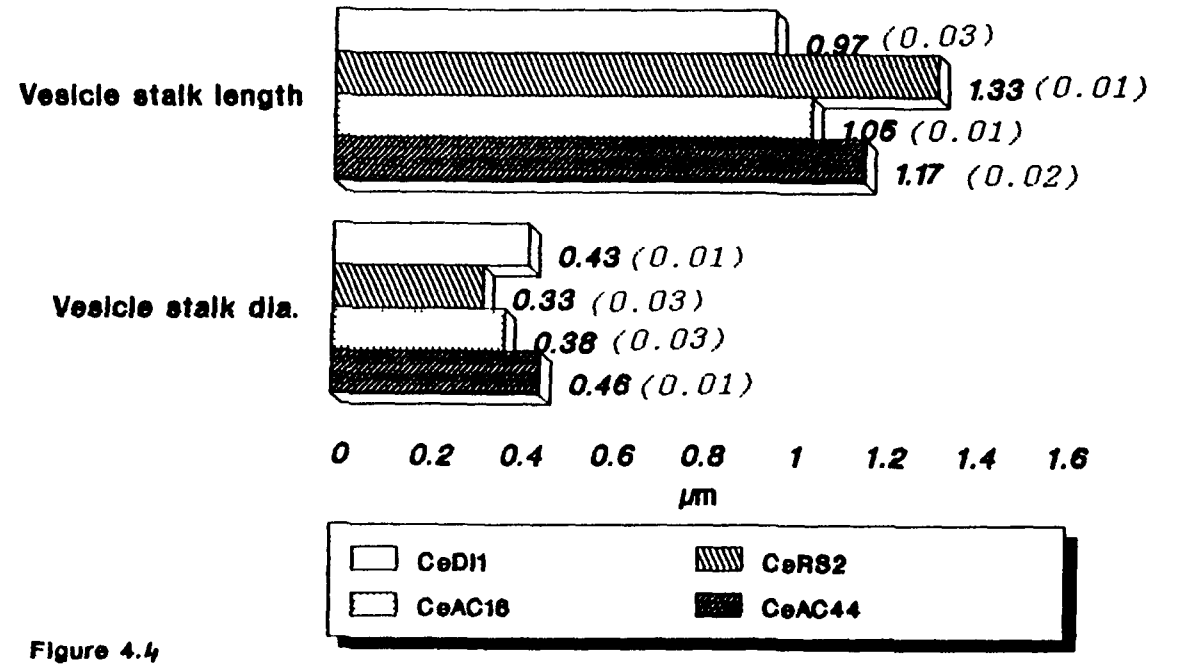


Figure 4.4

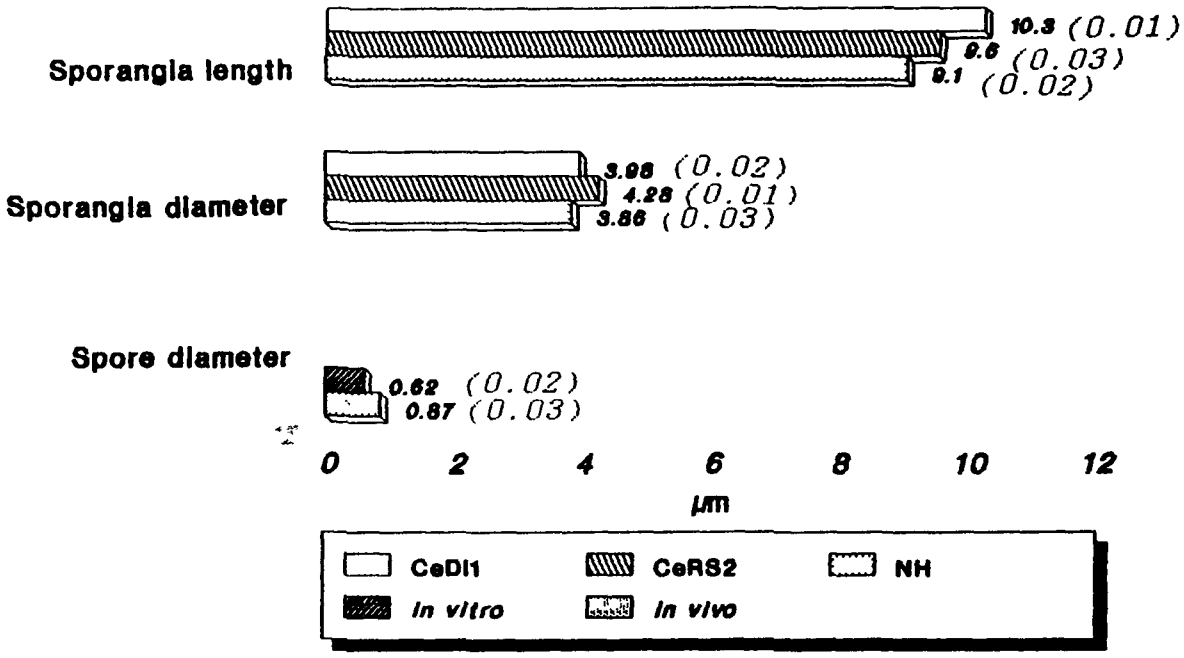


Figure 4.5

Figure:4.4 Mean of vesicle stalk length and vesicle diameter for four isolates studied. Figures in parentheses represent standard error of the mean.

Figure:4.5 Mean values of sporangia length and sporangia diameter for two isolates in which in vivo sporangia were seen and one field collected nodule (NH) from Hyderabad. A comparison of spore diameter *in vitro* (average values for both isolates) and *in vivo* (for the NH) is also presented. Figures in parentheses represent standard error of the mean.

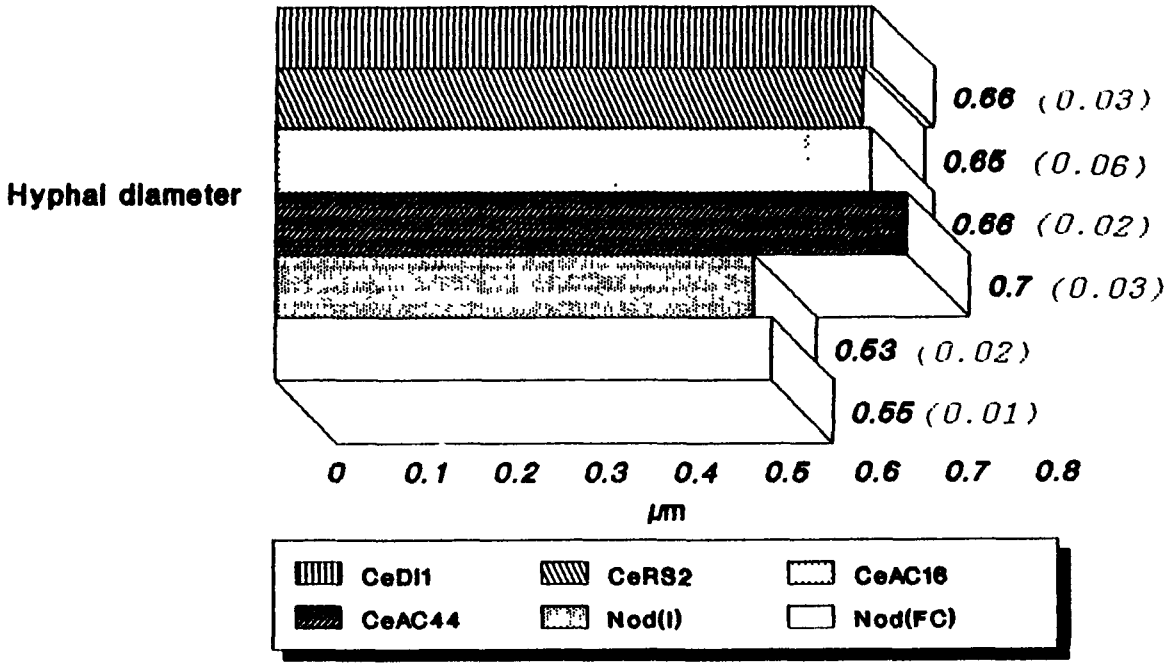


Figure 4.2

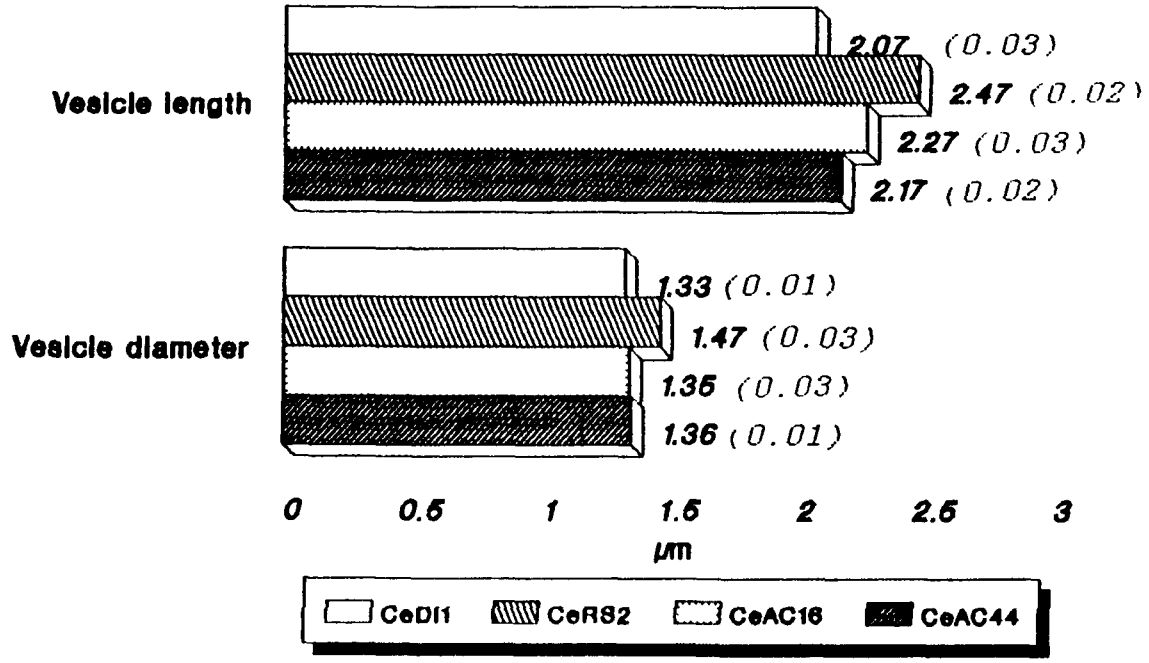


Figure 4.3

Figure:4.2 Mean hyphal diameter of four isolates and *in vivo Frankia* of induced and field collected nodules. Nod(I) represents mean of values in nodules induced by CeDI1 and CeRS2. Nod(FC) is for field collected nodules. Figures in parentheses represent standard error of the mean.

Figure:4.3 Mean vesicle length and diameter of four isolates studied. Figures in parentheses represent standard error of the mean.

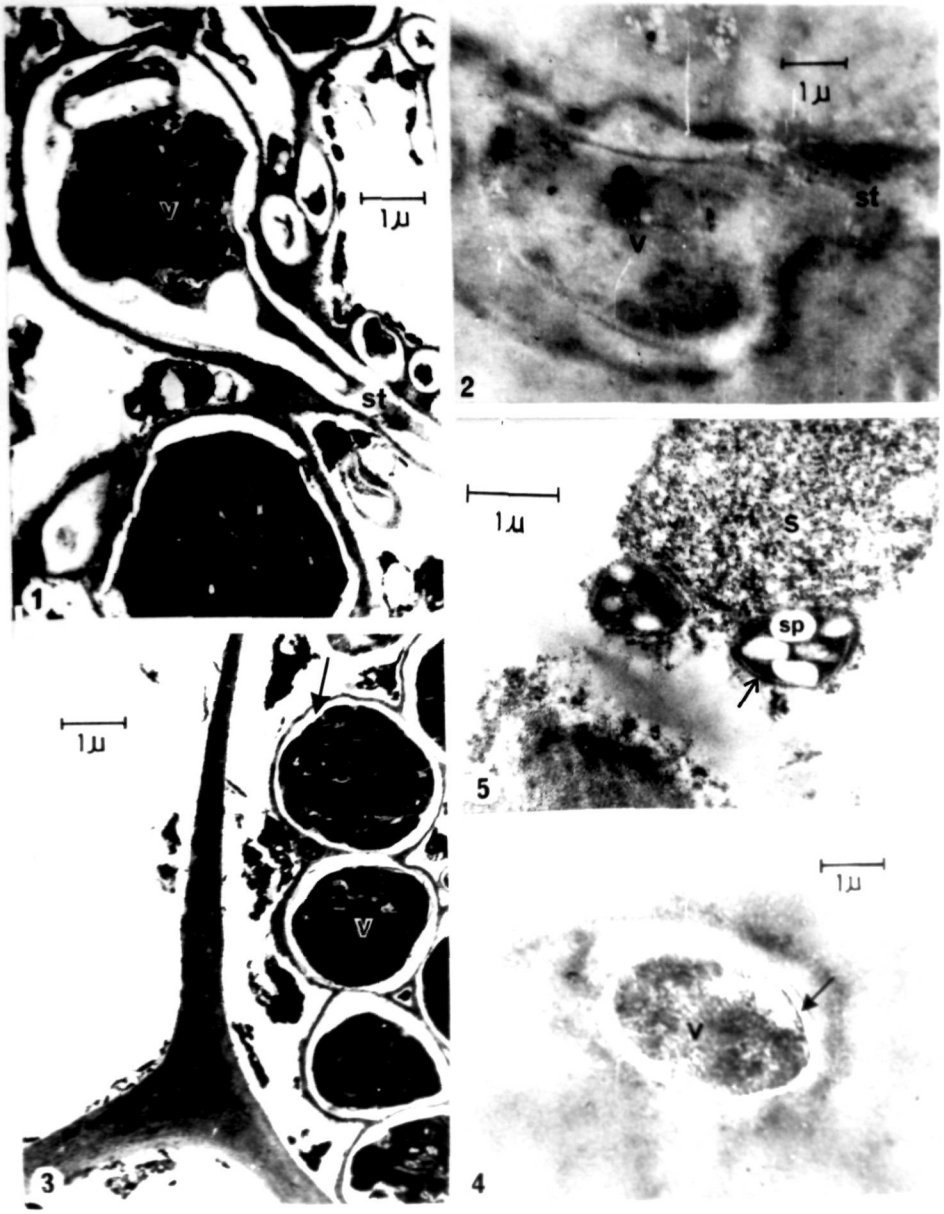


Plate E

Plate F

1. Close up view of plate E-3 showing the intravesicular septa as well as plant cell wall derived capsule (arrow).

2. Cross section of hypha(h) and vesicle(v) of CeRS2.

3. Transmission electron photomicrograph showing L S of vesicle(v) with septum (arrow).

4. Transmission electron photomicrograph showing T S of nodule cell with *Frankia* hyphae(h) inside.

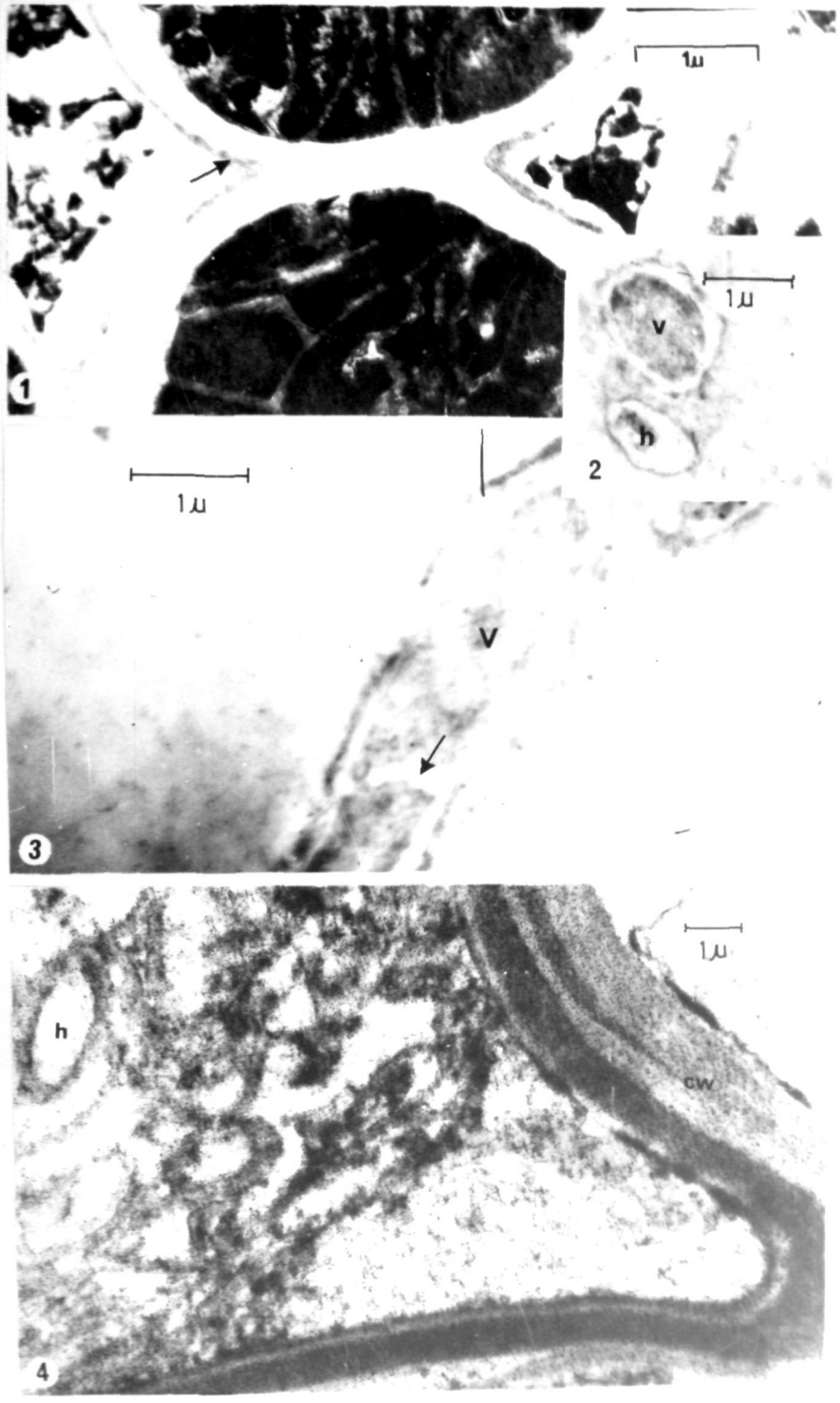


Plate F

4). The presence of the void area clearly demonstrated that this is present between the two layers of microsymbiont origin and not that of the plant and microsymbiont origin as suggested by Benson and Silvester (1993).

Freeze-fracture electron microscopy of actinorhizal nodules demonstrated that a portion of the symbiotic vesicle envelop was comprised of up to 30 laminae (Newcomb and Wood, 1987). Rapidly frozen and freeze substituted *Frankia* vesicles lack that large void area and also show a laminated structure in the cell wall (Lancelle *et al.*, 1985; Benson and Silvester, 1993). The lamina observed in rapidly frozen samples are missing in the chemically fixed tissues, suggesting that the lamina may be extracted during the chemical treatment to form the void area (Lancelle *et al.*, 1985). Chemical fixation including glutaraldehyde-osmium tetraoxide did not preserve the laminated portion of the cell wall of *Frankia* vesicles. In addition, acetone, used for dehydration during the preparation of TEM specimens probably dissolved the lipid portion of the laminae (Torrey and Callaham, 1982; Newcomb *et al.*, 1987) Saturated lipids do not react in a major way with

glutaraldehyde and osmium tetraoxide and thus did not become a part of the cross-linked preserved structure of fixed cells. The loss of these lipids may be at least partially responsible for the formation of the void area.

The significance of the laminated vesicle wall may be partly understood by examining the hetercysts of cyanobacteria which also have an envelop comprised of laminae which are also poorly preserved by glutaraldehyde-osmium tetraoxide fixation. In cyanobacteria, the laminae are made up of glycolipids. Mutants of *Anabaena* deficient in the glycolipids of the laminated heterocyst envelop were unable to fix nitrogen aerobically, but were able to do so when oxygen tension was reduced, thus suggesting that laminae play a role in protecting the nitrogenase from oxygen inhibition. Measurements of oxygen uptake by *Anabaena* also suggested that oxygen enters the heterocysts rather slowly compared to the vegetative cells. Since the site of nitrogenase activity is believed to be the symbiotic vesicles of *Frankia*, the laminated wall may have a similar equally important role regulating

oxygen availability but not creating a nitrogen deficiency because of the different concentration gradients of nitrogen and oxygen (Dixon and Wheeler, 1986). Kinetic analysis of oxygen uptake by cultured *Frankia* vesicles and hyphae provides evidences for a passive gas diffusion barrier in *Frankia* vesicles (Murry *et al.*, 1984).

Besides the void area, other changes in the appearance of preserved symbiotic vesicles due to fixation are possible. For example, differentiated vesicles may shrink during chemical treatments, giving a more electron dense appearance (Plate E 1). I have also noticed clear intra vesicular septa (Plate F-1) and the presence of septum between the stalk and vesicle (Plate F-3). These may be features for further enhancing the nitrogenase protection mechanism of vesicles.

4.5.3.3 Sporangia

As mentioned earlier, nodules collected from Hyderabad showed the presence of sporangia *in vivo*. Plate E-5 shows the LS

of sporangia (s) with sections of spores (sp). Mean diameter of the spores was 0.86μm. The spore had an outer electron dense membrane like layer and an electron transparent cell wall. Inside the spores, electron translucent granules were present, which may be reserved food material. As mentioned earlier, sp^+ strains are rare and have not been established in *ex planta* cultures so far. This behaviour has made scientists to classify them as a genetically separate group.

4.6 DNA BASE COMPOSITION OF *FRANKIA*

Becking's classification of *Frankia* according to their host plant is no more tenable after the cross infectivity has been proved (Bosco *et al.*, 1992; Benson and Silvester, 1993). Although serological (Baker *et al.*, 1981), chemical (Lechevalier and Lechevalier, 1979) and physiological (Blom *et al.*, 1980) studies have provided valuable information about the isolates, no unique characterization for use in classification has emerged from these studies (An *et al.*, 1983). In this context, DNA base composition seems to be useful for characterization of *Frankia*. Nucleoside

Isolates	G+C mol%
CeDI1	71.5
CeRS2	70.0
CeAC16	69.8
ACN1AG	71.0

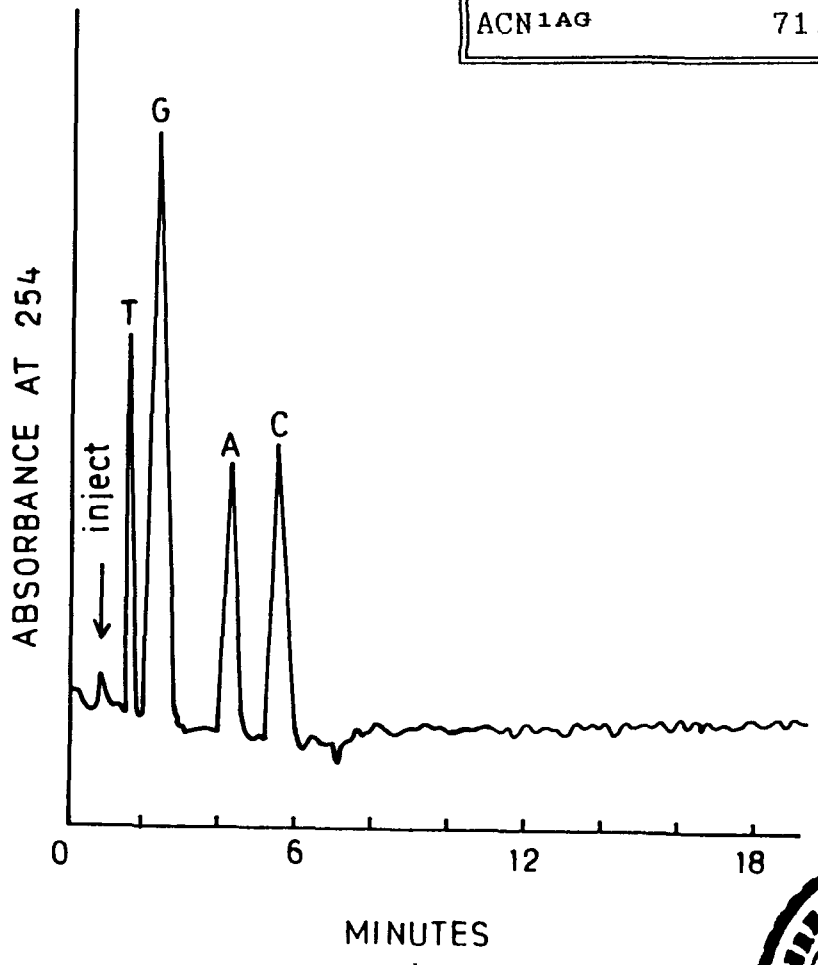


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Figure: 4.6 Chromatography on PepRPC of nucleosides from enzymatic hydrolysis of CeDI1 DNA. In the box: G+C mol% of all the three isolates and the reference strain.

analysis on PepRPC column of four endophyte isolates gave G+C values of 69.8 to 71.5 mol% (Fig 4.6). Out of these four isolates, three were isolated from *Casuarina equisetifolia* in our laboratory (CeDI1, CeRS2, and CeAC16). The 4th one was a reference strain (ACN^{1AG}), obtained from Lyon collection France.

According to Benson and Silvester (1993), the mol% of *Frankia* DNA ranges from 66-75% and my isolates had mol% well within this range. A higher G+C mol% confers higher radioprotection to DNA. Thus, *Frankia*, like many other prokaryotes, is endowed with the ability to withstand higher radio stress.

The isolates, under study were indistinguishable by their G+C content. Though it is not possible to draw positive quantitive conclusions about the degree of relatedness of the isolates from base composition alone, these results do provide qualitative information on the relatedness of these isolates to other actinomycetes, particularly actinomycetes of cell-wall type III, to which *Frankia* belongs (Lechevalier and Lechevalier,

1979). Although a similar base composition does not necessarily indicate a close genetic relationship, the less similar the composition, the less likely it is that the organisms are related. Theoretically, if the nucleotide compositions of bacteria differ by 18% G+C or greater, they have few if any common nucleotide sequences and thus have only a very distant evolutionary relationship (De Lay, 1969; An *et al.*, 1983).

4.7 ISOLATION OF GENOMIC DNA FROM *FRANKIA* CULTURES

Since *Frankia* grows very slowly in culture, an efficient lysis is a prerequisite for good yield of total genomic DNA. Chemical dissolution of the cell wall by hot lauryl sulfate (SDS) (Normand *et al.*, 1983), use of enzyme like lysozyme and achromopeptidase (Simonet *et al.*, 1984) and mechanical shearing of cell wall by ultrasonication etc. are some procedures employed for better lysis. I tried enzyme induced lysis. For the purpose, lysozyme and achromopeptidase alone or in combination were tried for cultures of different ages. The results have been presented in Table 4.3. Presence or absence of DNA was based on a visual

TABLE 4.3
Effectiveness of lysis using lysozyme and achromopeptidase enzymes singly and in combination

Age of cultures	Enzymes used	Results*
7-15 day	lysozyme	+
7-15 day	lysozyme + achromopeptidase	++
7-15 day	achromopeptidase	-
20-30 day	lysozyme	-
20-30 day	lysozyme + achromopeptidase	+
20-30 day	achromopeptidase	-

*Symbols: +sign denotes lysis, ++denotes better lysis, -denotes no lysis

observation on agarose gel (Plate G-1). It was found that if other conditions remained constant, 10mg/mL of lysozyme supplemented with a pinch of achromopeptidase gave best results. Achromopeptidase is a crude enzyme extracted from *Achromobactor lyticus* and has been successfully used with lysozyme to release more protoplasts from hyphae of *Streptomyces.* The present findings are in line with those of Simonet *et al.* (1984).

For the precipitation of DNA, both ethanol and isopropyl alcohol were used. The best results were obtained with the use of isopropyl alcohol.

4.8 ISOLATION OF *FRANKIA* DNA FROM THE NODULES OF *CASUARINA*

As already mentioned, isolation of *Frankia* in pure culture is a major problem in *Frankia* research That is why, until recently a major portion of *Frankia* research remained confined to isolates obtained in pure culture, and many more *Frankia* strains remained uncharacterized for want of suitable isolation techniques (Mirza *et al.*, 1991). But isolation of endophyte DNA directly from the nodule helps to perform PCR amplification, clon-

Plate G

Agarose gel electrophoresis of:

1. Isolated total genomic DNA from cultures-
   Lane 1. With lysozyme and Achromopeptidase (CeRS2)[1]
   Lane 2. With lysozyme and Achromopeptidase (CeAC16)[1]
   Lane 3. With lysozyme (CeRS2)[1]
   Lane 4. With lysozyme (CeAC16)[1]
   Lane 5. With lysozyme and Achromopeptidase (CeRS2)[2]
   Lane 6. With lysozyme and Achromopeptidase (CeAC16)[2]

([1]12 day old culture; [2]28 day old culture)

2. Isolated total nodule DNA using two different methods (see text)
   Lanes 1to3 Isolation using sonication method of Misra *et al.* (1991)

   Lanes 4to6-Isolation using the method based on Simonet *et al.* (1990)

3. Amplified 16S rRNA gene using primers MGL4 [FPGS 989ac of Bosco *et al.* (1992)] and MGL7.

   Lane 1. DNA isolated from CeDI1.
   Lane 2. DNA isolated from CeRS2.
   Lane 3. DNA isolated from nodules (Arunachal Pradesh)
   Lane 4. -ve control

4. Amplified *nif* region using primers MGL1 (*nif*D, coordinates 807) and MGL2 (*nif*K, coordinates 700').

   Lane 1. DNA isolated from nodules (Arunachal Pradesh)
   Lane 2. DNA isolated from CeAC16.
   Lane 3. DNA isolated from CeAC44.
   Lane 4. DNA isolated from CeDI1.
   Lane 5. DNA isolated from CeRS2.
   Lane 6. DNA isolated from ANC[1AG].
   Lane 7. -ve control

5. PCR/RFLP pattern of part of 16SrRNA gene using *Nci*1.

   Lane 1. Digested CeDI1 DNA
   Lane 2. Digested CeRS2 DNA
   Lane 3. Digested nodule DNA (A P)
   Lane 4. Control CeDI1 DNA
   Lane 5. Control CeRS2 DNA
   Lane 6. Control nodule DNA (A P)

L in each case represents molecular weight marker (Lamda DNA double digested with *Eco*R1 and *Hind*III).

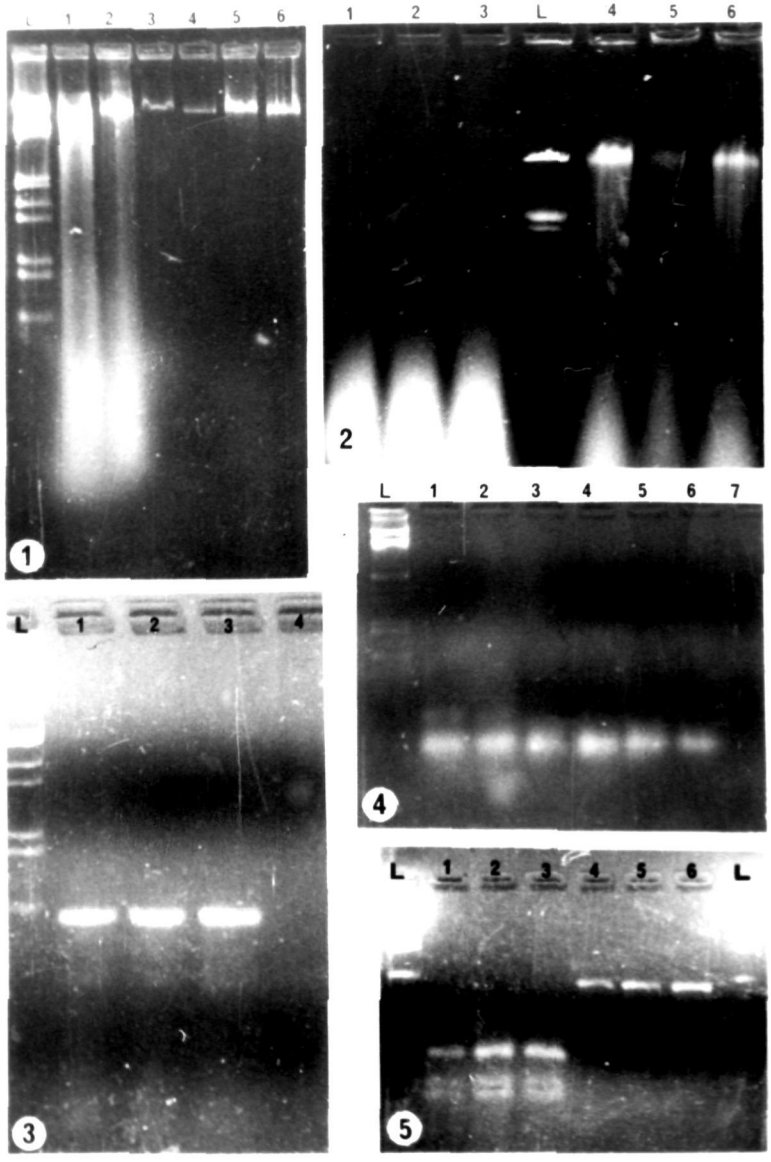


Plate G

ing, sequencing etc. of the uncultured strains with relative ease. Since the buoyant density of endophyte DNA is much greater (p=~1.72) than the buoyant density of host plant (p=~1.68), a method involving CsCl density gradient centrifugation was developed by Mulin *et al.* (1983). But CsCl density gradient centrifugation is a hazardous and tedious process. Misra et al. (1991) and Simonet et al. (1991) used an ultrasonication based protocol for isolation of endophyte DNA for subsequent use in PCR procedures. This procedure caused shearing of DNA, imposing a restriction on the length of DNA segments that could be amplified. Simonet et al. (1990) used a method based on lysis based on lysis induced by lysozyme and achromopeptidase, followed by lauroylsarcosine. I too used this protocol and got very good recovery (Plate G-2). For PCR amplification of *Frankia* DNA isolated directly from nodules using an isolation method described in 3.7.2 was found to be very useful. Although this method yielded very small quantity of DNA (some times no bands could be seen on agarose gels), it was good enough for amplification. In fact it

was found that too much DNA interferred with successful amplification and sometimes needed one thousand fold dilution before use.

4.9 PCR AMPLIFICATION

DNA isolated from *Casuarina* isolates (CeDI1 and CeAC16), as well as nodules collected from Arunachal Pradesh, was yielded, as expected, a ~500bp band upon amplification with a set of primers of which one was *Frankia* specific 16mer oligonucleotide primer (*Alnus* and *Casuarina* compatible, Bosco *et al.*, 1992) (Plate G-3). Another segment of *Frankia* DNA comprising of *nif*D-*nif*K IGS region was also amplified using the primers as mentioned in section 3.11, yielding 1400bp fragment, as expected (Plate G-4). The annealing temperature ($50^{o}C$) worked out for the primer pair based on Zeff and Geliebeter (1987) yielded multiple bands for primers for *rrn* region. However an increase in stringency of annealing ($55^{o}C$) resulted in a single expected band. It seems that at lower annealing temperature some nonspecific amplification occurred which was overcome on increasing the annealing temperature. A

similar type of specificity for other primers was discerned by Simonet *et al* (1991) The fact that I could achieve amplification for these two regions and also that *Frankia* genome specific primers yielded successful amplification indicated that my isolates were indeed *Frankia*. This further substantiated my earlier results on nodulation test and G+C contents.

For a meaningful comparison, I also amplified the same regions using DNA isolated from field collected nodules from Arunachal Pradesh. These too yielded the bands similar to the ones obtained for the isolates under study.

4.10 RFLP

I wanted to compare my isolates at molecular level. Since RFLP pattern is a very reliable method of such comparison, I decided to workout the RFLP pattern for the 16S rRNA region amplified as above. For this I chose restriction enzyme *Nci*I which was found to yield varying patterns (Rouvier, personal communication). The two isolates (CeDI1 and CeAC16) and nodule endophyte from Arunachal Pradesh, yielded similar patterns (Plate

G-5). Thus I was not able to detect any variability using this region and restriction enzyme combination. Probably, a more advantageous approach would have been the RFLP pattern covering the ITS of the *rrn* operon, because any variations arising in this region are likely to survive for obvious reasons.

4.11 CONCLUSION

In concluding, the following points are highlighted:

1. *Frankia* isolates from nodules of casuarina and alder were obtained.

2. A rapid technique for isolation through Ca-alginate beads was developed.

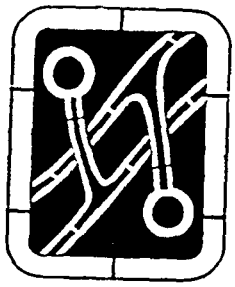
3. Nodulation tests, morphological and anatomical studies under SEM and TEM, G+C mol% content and DNA amplification using *Frankia* genus specific probes for 16S rRNA gene and *nif* genes confirmed the isolates to be *Frankia*.

4. One of the isolates had statistically significant higher

nitrogenase activity compared to the reference strains.

5. Marginal morphological variability was seen amongst the isolates.

6. No variability at the molecular level could be detected using restriction enzyme *Nci*1 for distal region of 16S rRNA gene.



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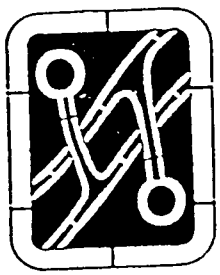

APPENDICES

APPENDIX I

Proceedings of International *Frankia*-Actinorhizal conference*

#	Year	Meeting place	Organizers	Publication
1.	Aug. 1978	Harvard Forest, USA	Torrey & Tjepkema	*Bot. Gaz.*
2.	--- 1979	Corvallis, Oregon, USA	Gordon *et al.*	*Oregon State Univ. Pre.*
3.	Aug. 1982	Madison, Wisconsin, USA	Torrey & Tjepkema	*Can. J Bot.*
4.	Sep. 1983	Noordwijkerhout, Neatherlands	Akkermans *et al.*	*Plant & Soil*
5.	Aug. 1984	Quebec, Canada	Lalonde *et al.*	*Plant & Soil*
6.	Aug. 1986	Umea, Sweden	Huss-Dannel & Wheeler	*Physiol. Plant.*
7.	Aug. 1988	Storrs, Connecticut, USA	Winship Benson	*Plant & Soil*
8.	Sep. 1991	Lyon, France	Normand *et al.*	*Acta Oecologica*
9.	Apr. 1993	Waikato, New Zealand	Silvester & Harris	*Soil Biol. Biochem.*
10.	Aug. 1995	Davis, USA	Berry & Myrold	*Physiol. Plant.*

* After Newcomb and Wood (1987) and Philippe Normand (personal communication).

Appendix II

Composition of culture media

DPM (N_2 free)

Constituents	Concentrated stock solution (per liter)	Working solution
(A) MACRONUTRIENTS	10X	
KH_2PO_4[1]	10.0 g	7348 μM
$MgSO_4$[2]	1.0 g	0899 μM
$CaCl_2.2H_2O$[2]	0.1 g	0068 μM
$C_3H_5O_2Na$[4]	12.0 g	12490 μM
(B) MICRONUTRIENTS	1000X	
H_3BO_3[3]	2.860 g	46.3 μM
$MnCl_2.4H_2O$[2]	1.810 g	9.1 μM
$ZnSO_4.7H_2O$[2]	0.220 g	0.8 μM
$CuSO_4.5H_2O$[2]	0.080 g	0.3 μM
Na_2MoO_4[2]	0.025 g	0.1 μM
$CoCl_2.7H_2O$[2]	0.025 g	0.1 μM
(C) IRON	1000X	
$FeSO_4.7H_2O$[1]	10.008 g	370.4 μM
Na_2 EDTA[3]	13.410 g	36.0 μM

pH—7.00

[1]HiMedia laboratories, Bombay, India
[2]E Merck (India) ltd. Bombay, India
[3]Qualigens fine chemicals Bombay, India
[4]Sigma chemical co. St. Louis, USA

F

Constituents	Concentrated stock solution (per liter)	Working solution
(A) MACRONUTRIENTS	10X	
K_2HPO_4[1]	5.0 g	2871 μM
$MgSO_4.7H_2O$[2]	2.0 g	0811 μM
$CaCl_2.2H_2O$	1.0 g	2127 μM
D-Glucose[3]	100.0 g	5550 μM
Hydrolysate casein[4]	40.0 g	
Yeast extract[1]	0.5 g	
(B) OLIGO QUISPEL	1000X	
H_3BO_3[3]	1.500 g	24.3 μM
$MnSO_4.H_2O$[2]	0.800 g	4.7 μM
$ZnSO_4.7H_2O$[2]	0.600 g	2.1 μM
$CuSO_4.5H_2O$[2]	0.100 g	0.4 μM
$(NH_4)_6Mo_7O_{24}.4H_2O$[2]	0.200 g	0.16 μM
$CoSO_4.7H_2O$[2]	0.025 g	0.09 μM
(C) IRON	1000X	
$C_6H_8O_7.H_2O$[1]	10.000 g	47.6 μM
$C_6H_8O_7Fe.3H_2O$[1]	10.000 g	33.5 μM
(D) VITAMIN	1000X	
Thiamine HCl[1]	0.010 g	0.03 μM
Nicotinic acid[1]	0.050 g	0.40 μM
Pyridoxine HCl[1]	0.050 g	0.24 μM
Tween 80[1]		1 mL
pH—7.00		

[1]HiMedia laboraties, Bombay, India
[2]E Merck (India) ltd. Bombay, India
[3]Qualigens fine chemicals Bombay, India
[4]Sigma chemical co. St. Louis, USA

Hoagland (modified)

Constituents	Concentrated stock solution (per liter)	Working solution
(A) MACRONUTRIENTS	10X	
KH_2PO_4[1]	1.36 g	999 μM
$MgSO_4$[2]	3.38 g	3039 μM
(B) MICRONUTRIENTS	1000X	
H_3BO_3[3]	2.860 g	46.3 μM
$MnCl_2.4H_2O$[2]	1.810 g	9.1 μM
$ZnSO_4.7H_2O$[2]	0.220 g	0.8 μM
$CuSO_4.5H_2O$[2]	0.080 g	0.3 μM
Na_2MoO_4[2]	0.025 g	0.1 μM
$CoCl_2.7H_2O$[2]	0.025 g	0.1 μM
(C) IRON	1000X	
$FeSO_4.7H_2O$[1]	10.008 g	370.4 μM
Na_2 EDTA[3]	13.410 g	36.0 μM

pH-7.00

[1]HiMedia laboratories, Bombay, India
[2]E Merck (India) ltd. Bombay, India
[3]Qualigens fine chemicals Bombay, India

OS-1

Constituents	Concentrated stock solution (per liter)	Working solution
(A) MACRONUTRIENTS	10X	
K_2HPO_4[1]	1.5 g	1102 μM
$NaH_2PO_4.2H_2O$[2]	1.3 g	833 μM
$MgSO_4.7H2O$[2]	0.5 g	202 μM
KCl[2]	1.0 g	1341 μM
$NaC_2H_3O_2.3H_2O$[1]	5.0 g	3658 μM
EDTA[3]	0.1 g	26.8 μM
Yeast extract[1]	2.5 g	
Tryptone[1]	2.5 g	
Tween 80[1]		1 mL
pH-7.00		

[1]HiMedia laboratories, Bombay, India
[2]E Merck (India) ltd. Bombay, India
[3]Qualigens fine chemicals Bombay, India

Qmod

Constituents	Concentrated stock solution (per liter)	Working solution
(A) MACRONUTRIENTS	10X	
K_2HPO_4[1]	3.0 g	1722 μM
$NaH_2PO_4.2H_2O$[2]	2.3 g	1474 μM
$MgSO_4.7H2O$[2]	2.0 g	0811 μM
KCl[2]	2.0 g	1682 μM
D-Glucose[3]	100.0 g	55506 μM
Yeast extract[1]	5.0 g	
Peptone[1]	50.5 g	
(B) MICRONUTRIENTS	1000X	
H_3BO_3[3]	1.500 g	24.3 μM
$MnSO_4.H_2O$[2]	0.800 g	4.7 μM
$ZnSO_4.7H_2O$[2]	0.600 g	2.1 μM
$CuSO_4.5H_2O$[2]	0.100 g	0.4 μM
$(NH_4)_6Mo_7O_{24}.4H_2O$[2]	0.200 g	0.16 μM
$CoSO_4.7H_2O$[2]	0.025 g	0.09 μM
(C) IRON	1000X	
$C_6H_8O_7.H_2O$[1]	10.000 g	47.6 μM
$C_6H_8O_7Fe.3H_2O$[1]	10.000 g	33.5 μM
(D) LIPID SUPPLEMENT	10X	
L-α-Lecithin[4]	0.400 g	

pH—7.00

[1]HiMedia laboratories, Bombay, India
[2]E Merck (India) ltd. Bombay, India
[3]Qualigens fine chemicals Bombay, India
[4]Sigma chemical co. St. Louis, USA

Appendix III

Composition of buffers

0.2M Cacodylate buffer (1X concentration)

197mM Cacodylic acid
(Dimethylarsinic acid, $Na(CH_3)_2AsO_2.3H_2O$)
Sodium salt[1]

Sodium cacodylate (42.2g) was dissolved in 800 mL of distilled deionized water. pH was adjusted to 7.4 with HCl and the volume was made up to 1000 mL with distilled water.

[1]Sigma chemical co. St. Louis, USA

DNA extraction buffer (1X concentration)

100mM M B grade Tris base[3] (pH 8.0 at 27°C)

20mM EDTA[2] (pH 8.0)

1.4M NaCl[1]

2% (W/V) CTAB[3]

1% (W/V) PVP[4]

Molecular biology grade Tris base (Trizma™ product # T8524, Tris[Hydroxymethyl]aminomethane, $C_4H_{11}NO_4$) (12.11g) was dissolved in 800 mL of distilled deionized water, pH was adjusted to 8.0. In a part of it, 7.44g EDTA was dissolved. In other part, 81.82g NaCl, 20g CTAB (Hexadecyl trimethylammonium bromide, $C_{19}H_{42}NBr$) and 10g PVP was added. Two parts were mixed together.

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[1]E Merck (India) ltd. Bombay, India
[2]Qualigens fine chemicals Bombay, India
[3]Sigma chemical co. St. Louis, USA
[4]SRL, Bombay, India

DNA loading buffer
(Type III, Sambrook *et al.*, 1989)

(6X concentration)

0.25% Bromophenol blue

0.25% Xylene cyanol FF

30% Glycerol in DD H_2O

Two and half grams of Bromophenol blue and Xylene cyanol was dissolved in 1000mL of 30% Glycerol.

1IDPL, Hyderabad, India
2S D fine chem, India

DNA wash buffer (1X concentration)

0.02M Tris.Cl (pH 7.2)

2mM EDTA (pH 8.0)

0.01M NaCl

Supplied as part of DNA cleaning kit.

Electrophoresis buffer (TBE)

(0.5X concentration)

0.045M Tris[2]-borate[1]

0.001M EDTA[1]

To prepare 5X stock soln:

Molecular biology grade Tris base (Trizma™ product # T8524, Tris[Hydroxymethyl]aminomethane, $C_4H_{11}NO_4$) (54g) and 27.5g boric acid were dissolved in 800mL of distilled deionised water. In it, 20 mL of 0.5M EDTA (pH 8.0) was added. pH was adjusted to 7.6 with conc. HCl. The soln. was dispensed into aliquots and was kept at room temperature.

TBE was originally used at a working strength of 1X for agarose gel electrophoresis. However, according to Sambrook *et al.* (1989), a working soln. of 0.5X provided more than enough buffering support. In this present study, I also found satisfactory results with 0.5X soln.

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[1]Qualigens fine chemicals Bombay, India
[2]Sigma chemical co. St. Louis, USA

GeneAmp™ PCR buffer

(1X concentration)

100mM Tris.Cl pH 8.3 (at 25°C)

500mM KCl

15mM $MgCl_2$

0.01% (W/V) gelatin.

Supplied with AmpliTaq™ DNA polymerase as a 10X concentrated stock and recommended for use with the same.

™AmpliTaq and GeneAmp are the trademarks of Roche Mol.Sys., USA.

Phosphate-buffered saline (PBS)

137mM NaCl[1]

2.7mM KCl[1]

10mM Na_2HPO_4[1]

1.8mM KH_2PO_4[1]

Eights grams of NaCl, 0.2g of KCl, 1.44g of Na2HPO4, and 0.24g of KH2PO4 were dissolved in 800mL of distilled deionised water. The pH was adjusted to 7.4 with HCl. Water was added to 1 liter. the soln. was distributed into aliquots and sterilized by autoclaving.

[1]E Merck (India) ltd. Bombay India

Tris-EDTA buffer (TE-8)

10mM Tris[2] (pH 8.0)

1mM EDTA[1] (pH 8.0)

Molecular biology grade Tris base (Trizma™ product # T8524, Tris[Hydroxymethyl]aminomethane, $C_4H_{11}NO_4$) (1.21g) was dissolved to 400mL of distilled deionized water and the pH was adjusted with conc. HCl to 8.0. and sterilized by autoclaving. Similarly, 0.372g Na_2-EDTA was dissolved in 400mL of distilled deionized water. The soln. was stirred vigorously and pH was adjusted with NaOH pellets and sterilizer by autoclaving. Both the soln. were then mixed and the volume was made up to 1 liter with D D water.

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[1]Qualigens fine chemicals Bombay, India
[2]Sigma chemical co. St. Louis, USA

Restriction enzyme buffer

50mM Potassium acetate

20mM Tris acetate

10mM Magnesium acetate

1mM DTT

pH 7.9 at 25°C

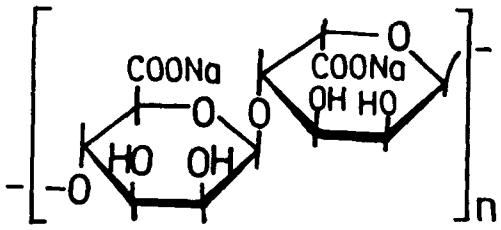
Supplied with restriction endonuclease *Nci*1 as 10X concentrated stock and recommended for use with the same.

Appendix IV

Structural formulae and necessary informations about some important chemicals used in this study

Alginic acid
Sodium salt

Unbranched copolymer consisting of residues of D-mannuronic acid and L-guluronic acid. Mainly used for immobilization of microorganisms. The alginate matrix can be liquefied by the addition of a chelating agent, making possible the recovery of developing colonies without affecting their physiology.

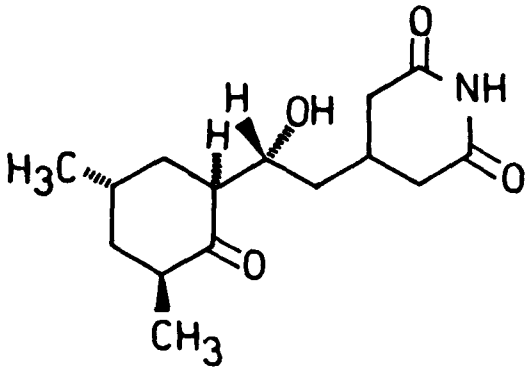


Cycloheximide
(Actidione)
(3-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide)

$C_{15}H_{23}NO_4$
M.W. 281.36

from *Streptomyces griseus*

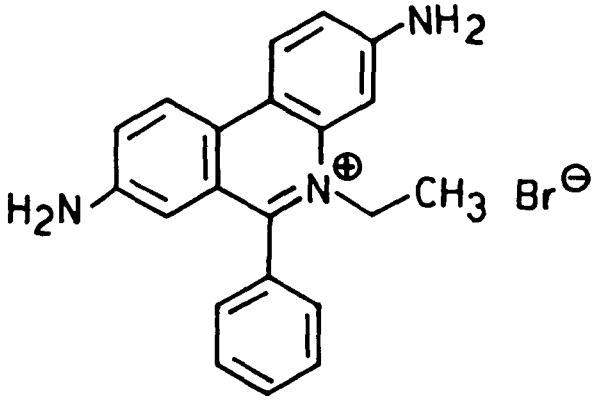
Inhibits protein biosynthesis in eukaryotic cells by binding with the 80S ribosome. Readily dissolved in ethanol.



Ethidium Bromide
(3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide; Homidium bromide)

$C_{12}H_{20}BrN_3$
M.W. 394.33

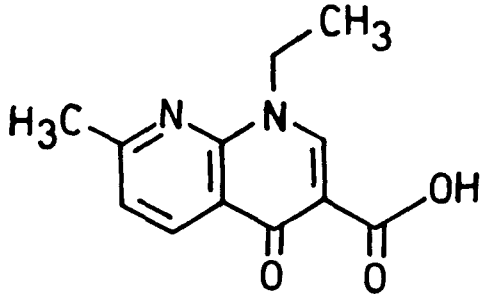
Nucleic acid intercalating agent. Solubility: 0.01g/mL of water.



Nalidixic acid
(1-Ethyl-1-4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid)

$C_{12}H_{12}N_2O_3$
M.W. 232.32

Inhibitor of bacterial DNA synthesis. Solubility: 1g/50mL chloroform.



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