USE OF MOLECULAR CHARACTERISTICS IN *FRANKIA* FOR STRAIN IDENTITY

ABSTRACT

By
GOPA SARMA

THESIS SUBMITTED
IN FULFILMENT OF THE DEGREE OF
DOCTOR OF PHILOSOPHY IN BOTANY

NORTH-EASTERN HILL UNIVERSITY
SHILLONG- 793 022, INDIA
1999

Thesis

ABSTRACT

ABSTRACT

Nitrogen is one of the most important elements for living organisms. Vast amount of nitrogen is available in the atmosphere, but the plants are unable to fix it for their use. Somehow this ability has been confined to micobes only. Actinomycete *Frankia*, is one of the microorganisms that fix atmospheric nitrogen. *Frankia* can enter into symbiotic association with some plants, called as actinorhizal plants. This association leads to the formation of root nodules.

Actinorhizal plants belong to eight families and 25 genera (Lechevalier, 1994). These actinorhizal plants have potential applications in reforestation and soil improvement. *Alnus*, *Hippophae* and *Elaeagnus* have been used for land stabilization, timber production and improvement of soil fertility.

Frankia are filamentous, branched, Gram positive bacteria. On the basis of the actinomycetous nature of *Frankia*, Becking (1970) placed them in the family Frankiaceae in the order Actinomycetales. In symbiotic association *Frankia* infect the cortical region of nodule tissue.

The isolation of *Frankia* proved difficult until the first reproducible isolation from *Comptonia peregrina* (Callaham *et al.*, 1978). In pure culture they have branched and septate hyphae ranging from 0.5-2.0 μM in diameter. The formation of sporangia, which are spherical in shape (3-4.05 μM diameter), is a characteristic feature of the genus *Frankia* (Lechevalier and Lechevalier, 1990). *Frankia* also produce swollen structures

called as vesicles. Vesicles are the site of nitrogenase activity, the enzyme responsible for nitrogen fixation (Meesters *et al.*, 1985). Vesicles are analogous to heterocysts of cyanobacteria because of their ability to restrict the diffusion of oxygen to the site of nitrogenase activity.

Frankia strains have been isolated from a large number of species within *Alnus* (Lechevalier, 1986). Extensive investigations on all aspects of *Frankia*-actinorhizal plant symbiosis have been taken up in the last fifteen years. Much of the work done on *Frankia* has been carried out using isolates generated by crushing nodules in suitable culture medium. In the present study also the nodules of *Alnus nepalensis* were collected from Arunachal Pradesh, India. The microorganism was isolated using the same method and its identity was confirmed. Our contention was that the very nature of this technique was likely to generate genetically mixed cultures. Unfortunately, very little work had been done with the view to purify the cultures genetically. Although Tzen *et al.* (1991), Prin *et al.* (1991) and Lumini and Bosco, (1996) carried out investigations on generation of single spore cultures, their attempts were confined to *Elaeagnus* and *Hippophae*. No reports were available on genetic purification of the *Alnus* infective *Frankia* cultures.

The Random Amplified Fragment Length Polymorphism (RFLP) technique has been used as a powerful tool to identify and distinguish closely related isolates (Bloom *et al.*, 1989). Restriction digestion of a given DNA fragment can give rise to a pattern of fragments that is informative of the location of target sites in the sequence. Mutation may lead to the change in the target sequence. DNA from different members of a genus were analyzed by such methods (Dobritsa *et al.*, 1985). Jamann *et al.* (1993) used such an approach for typing *Elaeagnus* infective *Frankia* strain.

The present investigations were taken up with the following objectives:

1. To obtain genetically pure cultures by generating single spore lines of some alder compatible *Frankia* strains.

2. To investigate the presence of variability in the original mother strain by using the molecular patterns and nitrogenase activities of the single spore lines derived from it.

3. Based on the variability observed, try to develop strain specific molecular markers.

For the present study the mother cultures were sporulated using 2µg/mL ampicilline. The sporulated cultures were filtered through 0.9µM glass filters and the spores were trapped on the glass filter. The filter was then shaken in fresh DPM medium and spore density counted on a haemocytometer. Appropriate dilution was done to achieve a spore density of 2 spores per drop of 5µL volume. This spore suspension was then mixed with 5% sodium alginate solution. The mixture was then dropped into 1% $CaCl_2$ solution. This resulted in the formation of alginate beads. The beads after hardening (by keeping them at

10°C) were transferred to fresh sterile medium and the isolates were obtained within two weeks.

To confirm the identity of *Frankia,* nodulation tests, nitrogenase activity studies and PCR with genus specific DNA primers were carried out. The results confirmed the single spore purified cultures to be *Frankia*.

The nitrogenase activity tests showed variation in respect to different single spore cultures. Further it was found that $AnpST11^{SSP1}$ was the best nitrogen fixer among the strains purified as above.

Variations with respect to amplification profiles using total 16S rRNA gene, distal part of 16S rRNA gene, 16S-23S *rrn* ITS, *nif* D-H IGS and *nif* D-K IGS were found both between the single spore cultures obtained from the same mother culture and those obtained from different mother cultures. Restriction Fragment Length Patterns were used to detect variation between different single spore purified cultures by digesting the amplified product with 4 base cutter enzymes. For 16S-23S ITS, *Nci*1 *and* for 16S rRNA gene *Scr*F1 were used. Prediction of the patterns as well as selection of the enzymes were done using MacVector software and Power Macintosh 6100/66 computer. It was observed that in majority of the cases of the digestion of 16S-23S ITS amplified DNA, three similar bands were observed. However, in case of $AnpUS8^{SSP1}$ there were four bands. Amongst that, one band matched with that of the *Casuarina* compatible strain ORS020606. When the amplified DNA for the distal part of 16S rRNA gene was digested, it was observed that all the other isolates showed similar patterns except AnpUS8^{SSP1}, where an extra band was present. This extra band was also similar to that of *Casuarina* compatible strain ORS020606.

Therefore, it was concluded that:

1. A novel technique for genetic purification of *Frankia* cultures was devised and tested. The genetic purification of mother strains by generation of single spore cultures was successful.

2. These single spore cultures were confirmed to be *Frankia*.

3. Nitrogenase activity assays, Amplification profile studies and PCR-RFLP studies confirmed the presence of variability among the single spore cultures derived from the common mother culture.

   So, the contention that the original cultures were genetically impure was right.

4. PCR-RFLP pattern specific to single spore purified culture AnpUS8^{SSP1} was identified.

   Thus, it is possible to use this approach for developing molecular signatures for genetically purified better performing *Frankia* strains.

USE OF MOLECULAR CHARACTERISTICS IN *FRANKIA* FOR STRAIN IDENTITY

By

GOPA SARMA

THESIS SUBMITTED
IN FULFILMENT OF THE DEGREE OF
DOCTOR OF PHILOSOPHY IN BOTANY

NORTH - EASTERN HILL UNIVERSITY
SHILLONG - 793 022, INDIA
1999

Thesis

The North-Eastern Hill University

April, 1999

DECLARATION

I, Gopa Sarma, hereby declare that the subject matter of this thesis entitled "Use of molecular characteristics in *Frankia* for strain identity" is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to any body else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the North-Eastern Hill University for the award of the degree of Doctor of Philosophy in Botany.

13.4.99
(Gopa Sarma)

(Head of The Department)
Head
Department of Botany
School of Life Sciences
N. E. H. U. Shillong-14

(Supervisor)

ACKNOWLEDGEMENTS

It gives me immense pleasure to place my profound indebtedness to Dr. Arvind K. Misra, Department of Botany, North-Eastern Hill University, Shillong, for his considered guidance, unwavering enthusiasm, untiring efforts and the much needed critical comment of his, to draft the manuscript. His inspiring encouragement was always a moral booster for me.

I shall be failing in my duty, if I do not express my gratefulness to Professor H. N. Pandey, Head, Department of Botany, NEHU., and Prof. P. Tandon and Prof. R.R. Mishra, formerly Heads for their kind permission to use laboratory facilities.

I express my sincere gratitude to all my respected teachers of the Department of Botany for their unconditional help and co-operation during the entire study period.

I wish to record my thankfulness to Dr. G. Ganesh and Dr. Arnab Sen for their ungrudging help and affection. Words are inadequate to express my appreciation to my lab mates Ms. Vineeta Chauhan and Mrs. Rajani Jacob.

I am most appreciative of the encouragement of Ms. Sanghamitra Purkayastha, Mr. Hiranjit Choudhury and Dr. Tapas Datta.

Mr. Subhasish Das Gupta, Mr. Babu John, Ms. Laxmi Prabha, Mr. Chittaranjan Deb, Ms. Lipika Das, Mr. Sanjiban Goswami, Mr. Atanu Bhattacharjee, Dr. Sanghamitra Chakraborty, Mrs. Madhabi Palit, and Ms. Deepika Das deserve my heartful commendation for their invaluable help at various stages of this study. I am also obligated to all my fellow hostel-mates for their help and cooperation.

Help received from the office staff, library, Mr. C. Marak (Lab Assistant) and especially Mr. P. B. Das, Store keeper of the Department of Botany are deeply acknowledged.

I remain evergrateful to my revered parents and my sister for their forbearance, affectionate concern and unsolicited support during the study period.

I am also thankful to many other friends and colleagues who helped in various ways and whose name may not appear here.

Last but not the least, financial support received from the project IFCPAR and DST in the form of research projects is gratefully acknowledged.

Sarma 13.4.99
(Gopa Sarma)

Shillong
Dated: 13.4.99

CONTENTS

LIST OF TABLES

APPENDICES

INTRODUCTION

Chapter 1

INTRODUCTION

Nitrogen is one of the most limiting nutrients for enhanced bio-productivity in natural and artificial ecosystems. The nutrient need of the growing world population depends chiefly on the success of modern agriculture, which to a large extent is regulated by the availability of nitrogen as chemical fertilizer. The supply of the chemical nitrogen fertilizer is dependent on fossil fuels and the cost of such chemical nitrogen fertilizers is galloping with a rise in the cost of fossil fuels. Extensive use of chemical fertilizers in agriculture for last few decades has caused pollution of aquatic and terrestrial ecosystems by rendering them eutrophic. This problem led scientists to work on developing alternative sources of nitrogen for agricultural use.

J.B. Boussingault (1838) first showed that legumes could obtain nitrogen from air. Hellriegel and Wilfarth were first to discover symbiotic nitrogen fixation in peas and clovers. The practice of including a legume in crop rotation system of farming seems to have been introduced in England by Sir Richard Weston, who was impressed by the benefits of clover in cropping sequences. On the basis of this experience, crop rotation spread to Europe, where food production increased by 40-50% during the eighteenth century. The search for the reason for the benefits of legumes became a subject for scientific study early in the development of agriculturalchemistry. Hellriegel and Wilfarth's pioneering work in later part of nineteenth century provided conclusive evidence on the involvement of legume root nodules in fixation of atmospheric nitrogen.

The atmospheric nitrogen is reduced by an enzyme called as nitrogenase. Nitrogenase is a complex molybdo-iron enzyme. It consists of two proteins, one of molecular weight around 220,000D, and the other around 68,000D (the exact weights depending on their bacterial sources). The larger unit contains two molybdenum atoms. It contains the site on which dinitrogen is reduced. The smaller one is the electron carrier.

All nitrogen fixing organisms are prokaryotes except possibly *Eriphorum vaginatum* (Chapin III *et al.*, 1993). Nitrogen fixing prokaryotes comprise both free living diazotrophs and symbionts that live in association with plants. A few free living bacteria like, *Clostredium pasteurianum, Klebsiella pneumoniae,* etc. fix nitrogen present in the atmosphere. Many cyanobacteria produce a characteristic cell called as heterocyst, which has hyaline contents and a thick, refractive wall. The firm identification that heterocysts are the site of nitrogen fixation and of the nitrogenase activity was given by Stewart *et al.* (1969). Bacteria of the genus *Rhizobium* fix atmospheric nitrogen in symbiotic association with roots of leguminous plants. The resulting symbiosis occurs in a specialised organ called as nodule. Diverse woody dicotyledonous plants have established symbiosis with an actinomycete called *Frankia*. *Frankia* sp. nodulated plants belong to about 200 species belonging to 25 genera distributed among 8 families of dicot woody plants some well known genera of actinorhizal plants are *Alnus*

(Alder), *Myrica* (Bayberry), *Hippophae* (Seabuckthorn), *Elaeagnus* (Autumn olive) and *Casuarina* (Beef wood).

The genus *Frankia* consists of slow growing actinomycetes with a doubling time of 50-60h (Diem *et al.*, 1989). The isolation of *Frankia* proved difficult for many years, until the first reproducible isolation from *Comptonia peregrina* was reported (Callaham *et al.*, 1978). Since then hundreds of new isolates have been obtained from several plant species in pure culture (Benson and Silvester, 1993). In culture, they have branched hyphae ranging from 0.5-2.0 μM in diameter. The formation of sporangia, which are spherical in shape (1-2 μM diameter) is a characteristic of the genus *Frankia* (Lechevalier and Lechevalier, 1990). In nitrogen free media, lipid encapsulated spherical vesicles (2-6 μMin diameter) are formed. They are attached terminally or laterally to the hyphae. These vesicles are the site of nitrogenase activity (Meesters *et al.*, 1985).

Alnus represents the actinorhizal genus for which the largest amount of information concerning variability at the intra- and interspecific level is available. *Frankia* strains have been isolated from a large number of species within *Alnus* (Lechevalier, 1986). Extensive investigations on all aspects of *Frankia*-actinorhizal plant symbiosis have been taken up in the last fifteen years. Much of the work done on *Frankia* has been carried out using isolates generated by crushing nodules in suitable culture medium. Our contention is that the very nature of this technique is likely to generate gentically mixed cultures. Unfortunately, very little work has been done with the view to purify the cultures genetically. Although Tzen *and Torrey.* (1989), Prin *et al.* (1991) and Lumini and Bosco

(1996) carried out investigations on generation of single spore cultures, their attempts were confined to *Elaeagnus* and *Hippophae*. No reports were available on genetic purification of the *Alnus* infective *Frankia* cultures.

The present investigations were taken up with the following objectives:

1. To obtain genetically pure cultures by generating single spore lines of some alder compatible *Frankia* strains.

2. To investigate the presence of variability in the original mother strain by studying the molecular patterns and nitrogenase activities of the single spore lines derived from it.

3. Based on the variability observed, try to develop strain specific molecular markers.

REVIEW OF LITERATURE

Chapter 2

REVIEW OF LITERATURE

2.1. THE ACTINORHIZAL SYMBIOSIS :

Actinorhizal root nodules were first described by Meyen (1829) in alder (*Alnus glutinosa*). Microscopic studies of those nodules led him to the conclusion that they were parasitic plants growing on the roots. They were called abnormal outgrowth of plants by Schacht (1853) and an out growth caused by insect bite by von Jäger (1860).

First root nodules in case of actinorhizal plants were described by Oersted (1865). Woronin's (1866) work was a great step towards the discovery of the actinorhizal nodule forming microorganism called *Frankia*. He described the anatomical structure of the root nodule in case of *Alnus glutinosa* which posses a central cylinder surrounded by a thick parenchymatous cortex. The tips of the intercellular hyphae form vesicular swellings in side the cells. These, he thought, were sporangia and therefore, named the microorganism as *Schinzia alni,* on the basis of a presumed resemblance to the parasitic fungus *Schinzia cellulicola.*

Gravis (1879), observing certain intercellular structures in alder root nodules, thought that a myxomycete, *Plasmodium*, was responsible for the root nodule formation. Möller (1885) too thought the same.

Woronin (1866) in his classical paper made extensive comparison between nodules of alder and Lupins and concluded that these two types contained different microorganisms. His idea was objected by Frank (1887) who considered them as protein deposits.

In 1886-1888 Brunchorst again studied the cytology of root nodule of leguminous plants and *Alnus* and was convinced that both of them were quite different from each other and in case of *Alnus* root nodule, a microorganism was surely involved. He considered them as fungi and thought that they were quite different from any myxomycetes. He named them as *Frankia subtilis*. This work led Möller to change his view and he proposed the name *Frankia brunchorstii* for the microorganism. After the magnificent work of Hellrigel and Wilfarth (1888) it was clear that there was difference between nitrogen-accumulating and nitrogen fixing plants, and plants like *Alnus* belonged to the second group. After observing the bacteriod formation, they also concluded that the microorganisms responsible for root nodule formation were bacteria and not fungi. Beyerinck (1888) first isolated the bacteria from root nodule of leguminous plant and concluded that they infected only leguminous plants.

Shibata (1902) was first to consider the microorganism of *Alnus* and *Myrica* as Mycobacterium after studying the cytology of the root nodule of these plants. A later paper by Shibata and Tahara (1917) included *Elaeagnus*, *Coriaria* and *Casuarina* in the same group as above by giving the evidence for an actinomycete like organism present in all nodules. Definite proof for the microsymbiont to be actinomycete was obtained after successful isolation of the microsymbiont. Electron microscopy of the microsymbiont confirmed its actinomycetes nature and Becking (1970) renamed it *Frankia* by reestablishing the old name Brunchorst had suggested.

Meanwhile work for isolation of *Frankia* in culture was going on, including that of Quispel (1954). In 1954 Pommer could isolate *Frankia* in simple nutrient medium containing glucose but unfortunately his cultures were lost. At last Callaham *et al.* (1978), after using Quispel's method for preparation of inoculate, succeeded in isolating and cultivating the microsymbiont from root nodules of *Comptonia peregrina* and the culture was named as CPI1.

2.2. THE HOST PLANT:

Actinorhizal root nodules are nitrogen-fixing symbioses involving the actinomycete *Frankia* and roots of dicotyledonous plants belonging to at least 8 plant families and 25 genera (Lechevalier M.P., 1994). These plants are diverse but have some common features. For example, all are perennial dicots and all except *Datisca*, are woody shrubs or trees. *Datisca* has a herbaceous shoot (Baker and Schwintzer, 1990).

The symbiosis of actinomycete and the host plant was known as non leguminous nitrogen-fixing symbiosis until 1978. In a meeting at Harvard Forest in Petersham, Massachusetts in 1978, the term 'actino' was given to the actinomycete *Frankia* and `rhiza' for the plant root, bearing the microsymbiosis. The microsymbiont was called as 'endophyte'. But at the 7th International meeting of

Frankia in 1988, it was decided to discontinue the term endophyte as it implied that the frankiae were plants and not bacteria.

Ecologically, majority of actinorhizal plants are pioneers on nitrogen poor, open sites. They are found in sandy and gravely sites, shores of streams, lakes, and wet lands (Baker and Schwintzer, 1990).

In India the actinorhizal plants are found in the coastal areas of south, the hilly areas of north-eastern region and some parts of Himalayas.

2.3. USES OF ACTINORHIZAL PLANTS:

Actinorhizal plants are mainly used to improve the fertility of degraded soils and peat bog bottom by increasing nitrogen and organic matter in soils. Economically these plants are used as timber and fuel wood and in forestry, for biomass production, reclamation and reforestation (Diem and Dommergues, 1990).

Alnus glutinosa has been extensively used fore mine spoil reclamation in Britain. *Elaeagnus* and *Hippophae* have been widely used for land stabilisation. *Alnus rubra* plants are extensively used for pulp and timber and fuel production (Dawson *et al.*, 1990). They are also planted as wind breaks and to stabilise dunes against wind erosion (Diem and Dommergues, 1990). Actinorhizal plants are also valued for landscaping, where they provide shade and contribute to the beautification of the parks and cities. Alder is used for preparation of apple chests in Himachal Pradesh. In Eastern Europe and China, *Hippophae rhamnoides* is cultivated for its fruits (Wheeler and Miller, 1990).

Although there are few actinorhizal plants in the tropics and subtropics, several species of *Casuarina* are planted extensively as wind breaks and to stabilise dunes against erosion (Diem and Dommergues, 1990). Actinorhizal plants have been used in traditional Indian medical systems for thousands of years.

2.4. THE SYSTEMATIC OF *FRANKIA*

Actinomycetes are filamentous, branching, gram positive bacteria. Most are free living saprophytes. Becking (1970), after years of trying to isolate the actinorhizal microsymbiont, decided that they were obligate symbionts and proposed a classification system based on cross inoculation studies on the pattern of *Rhizobium*. After the first isolation of *Frankia* in pure culture by Callaham *et al.* (1978), it was seen that the isolate was not species specific. Some time the isolate did not re-infect the host from which it was isolated although it infected other hosts belonging to different genus. On the basis of the actinomycetic nature of *Frankia*, Becking (1970) placed it in the family Frankiaceae in the order Actinomycetales. On the basis of physiology, two supra-generic and sub-generic groups (type A and B) were proposed by Lechevalier and Lechevalier (1989). Type A were the slow growers; like saprophytic actinomycetes. Type B were microaerophylic and could infect the host from which they were isolated. According to Stackebrandt (1985), there were two types of *Frankia*; A and B. He did the classification on the basis of 16S rRNA sequence studies. The constant regions of the 16S rRNA nucleotide sequence of actinomycetes have been used for determining the genera within the order Actinomycetales and for discerning the position of order

Actinomycetales under Prokaryote (Normand *et al.*, 1996). After the isolation of different strains of *Frankia* from different plants, Lalonde *et al.* (1988) proposed to name the species of *Frankia* according to their host specificity (*Frankia alni, Frankia elaeagnae*, etc.). For *Frankia alni* sub species name given was *pommerii* and *vandijkii* for Sp+ and Sp- strains respectively (Lalonde *et al.*, 1988). Normand *et al.* (1996) observed diversity among the *Frankia-Alnus* infective strains and divided them into three different groups. They proposed that the Frankiaceae family should be emended, to include only the genus *Frankia*. *Geodermatophilous* and *Blastococcus* should be excluded.

2.5. *FRANKIA* IN SYMBIOSIS:

In symbiotic associations, *Frankia* inhabits the cortical region of the nodule tissue. In most actinorhizal root nodules, the infected cells are uniformly distributed within the cortical region around a central stele (Benson and Silvester, 1993). In nodules of *Coriaria* and *Datisca*, infected cells are present on one side of the stele, resulting in asymmetric nodule structure in transverse section (Hafeez *et al.*, 1984; Newcomb *et al.*, 1982).

2.6. *FRANKIA* IN CULTURE:

Frankiae have been cultured only since 1978 after the isolation of first strain CPI1 from *Comptonia peregrina*. Once *Frankia* is isolated it grows well on simple media containing major salts and single carbon source (Callaham *et al.*, 1978; Baker and O′ Keefe, 1984).

2.7. MORPHOLOGY AND PHYSIOLOGY OF *FRANKIA:*

Three different cell types are observed in *Frankia* (Schwintzer, 1990). These are vegetative cells or filamentous mycelium forming hyphae on which the other two types of cells form the sporangia and the vesicles. The diameter of hyphae is between 0.5 to 1.5 µM. Sporangia bearing spores are produced *in vitro*. However, *in planta* formation of sporangia is seen in nodules of some genera. It has been observed that the environment within the nodule determines whether or not spores are to form (Torrey, 1987). However, the genotype of *Frankia* also plays a role. Depending upon presence or absence of spore formation, the strains are called Sp+ or Sp-. The Sp- strains sporulate when they grow in pure culture (Burgraff *et al.*, 1981, Baker, 1982; Normand and Lalonde, 1982).

According to Simonet *et al.* (1994), no one had succeeded in isolating in pure culture a typical Sp+ strain. Two hypotheses were proposed to explain this phenomenon.

According to first one, physiological conditions (pH, soil water condition, and age of plant) inhibit or stimulate sporulation in *Frankia* (Schwintzer, 1990). According to the other, more than one strain is present in a single nodule (Reddell *et al.*, 1985). There was evidence for the latter and it was seen that sporulation was determined by the genotype of the microsymbiont (Torrey, 1987).

Vesicles are analogous to heterocysts in cyanobacteria because of their ability to restrict the diffusion of oxygen to the site of nitrogenase activity. Vesicles are of three types, in case of *Alnus* and *Elaeagnus* they are spherical, in case of *Casuarina* and

Comptonia they are club shaped (Lalonde, 1979), and in case of the members of Rosaceae, *Cercocarpus* and *Dryas* they are elliptical or filamentous. Internally they may be septate or non septate. In *Casuarina* N_2 fixation takes place without vesicle formation. A specialized host cell-wall layer with hydrophobic properties restricts the penetrance of oxygen.

2.8. THE INFECTION PROCESS:

Many *Frankia* strains tested are known to be specific towards one particular host. It is possible to distinguish *Frankia* on the basis of their host specificity (Normand *et. al.*, 1996). The root nodule formation in case of *Frankia* are established through a series of interactions between *Frankia* hyphae and host root cells.

The infection process begins in the rhizosphere. Root hair of the plant secretes mucilage in which *Frankia* hyphae get embedded. When endosymbiont penetrates the host roots, the root hair get deformed. The tip of the root hair start multiplying. *Frankia* hyphae associated with deformed root hair penetrate at the site of folding of the root hair cell wall. The microsymbiont gets surrounded by host Plasma lemma. In *Alnus* multilamellar secondary wall deposition is observed only in infected root hair (Berry and Sunell, 1990).

2.9. THE GENETICS AND MOLECULAR CHARACTERISTICS OF *FRANKIA*:

The availability of cultures of *Frankia* has made it possible to examine the physical properties of the *Frankia* genome and to compare its genome to that of the other actinomycetes. Nucleoside analysis of *Frankia* revealed G+C % of 72% (An *et al.*, 1983) and it was almost similar to the value obtained for other actinomycetes. Relatedness amongst different *Frankia* strains were studied and it was observed that the DNA-DNA hybridization could not show any significant relatedness between different *Frankia* strains (An *et al.*, 1985). However, Farnandez *et al.* (1989) studied 43 isolates based on their DNA relatedness and could differentiate nine genomic species. Among Fernandez's genomic species groups, 3 genomic species were found compatible with the members of the genus *Alnus*. Genomic species 1 corresponded to gene group 1 of An *et al.* (1985). She obtained five genomic species compatible with members of the genus *Elaeagnus* and one genomic species among strains compatible with members of genus *Casuarina*.

The confusion regarding the relatedness of two different *Frankia* strains and other actinomycetes led scientists to study 16S ribosomal RNA genes. 16S rRNA genes are frequently used molecular chronometers. The sequence of these genes are more or less conserved throughout the living kingdom. A sequence variation represents an evolutionary transition. There are regions of large variability and large sequence conservation. The conserved regions indicate close relationship among the various genera and the variable regions are used to characterize species or even sub-species specific probes (Ganesh *et al.*, 1994). So the primers for different regions were designed and the genomic DNA were amplified by PCR method using such primers.

2.9.1. DESIGNING OF UNIVERSAL PROBES IN THE 16S rRNA GENE:

The different regions of 16S rRNA have been used to construct suitable primers for *Frankia* (Simonet *et al.*, 1991). 16S rRNA oligonucleotide analysis using different synthetic primers was used to determine the phylogenetic relationships among actinomycetes including *Frankia* (Hahn *et al.*, 1989). Primers FGPS1176' and FGPS 1490' (Nazaret *et al.*, 1991) were constructed from this region. 16S rDNA is also used to characterize universal primers. Primer FGPS 849 was used in conjunction with FGPS 1176' (Nazaret *et al.*, 1991) and it could amplify a 327 base pairs (bp) fragment in *Frankia* strain ORS 602606 (Normand *et al.*, 1992-a). Using reverse transcriptase or oligonucleotide cataloging of 16S rDNA it was found that a close phylogenetic relationship did exist between members of genera *Frankia, Geodermatophilous,* and *Blastococcus* (Simonet *et al.*, 1991).

To differentiate between two different *Frankia* strains obtained from different groups of actinorhizal plants, *Frankia* specific primer FGPS 989ac and FGPS 989e were designed which were specific in helix 31 of domain III of 16S rDNA gene (Bosco *et al.*, 1992). While FGPS 989ac was specific to *Frankia* isolated from *Alnus* or *Casuarina*, FGPS 989e was specific to *Frankia* isolated from *Elaeagnus*.

2.9.2. PRIMERS FOR 16S-23S ITS REGION:

Simonet *et al.* (1991) designed the primer based on a test on the size of the ITS separating 16S and 23S rDNA genes, in order to differentiate between the genera *Frankia* and *Geodermatophilous*.

The expected band sizes of the amplified products were different for the genera and the primers failed to amplify the DNA of other organisms.

2.9.3. DESIGNING UNIVERSAL PRIMER IN THE *nif* REGION:

The *nif* genes coding for the structure of the nitrogenase enzyme are also used to study the phylogenetic relationship. Nitrogen fixation is a characteristic feature of *Frankia*. Simonet *et al.* (1988), after digestion of total DNA and sub cloning of *nif* K and *nif* D genes of strain ArI3, concluded that the organization of the *Frankia* nitrogenase gene region was similar to that of *K. pneumoniae, R. meliloti* or *B. japonicum* where the *nif* AB genes were found to be separated from *nif* HDK. The nif sequences also have both conserved and variable regions like 16S rRNA gene and have been used for specific detection of *Frankia* strains by PCR (Simonet *et al.*, 1990).

The *nif* D-K IGS (Intergenic spacer) is more appropriate to characterize strains than the *nif* H-D IGS (Jamann *et al.*, 1993), because of its size and amount of variability (Normand *et al.*, 1992-b). FGPH 750 and FGPD 913′ (Simonet *et al.*, 1991) were designed for amplification of *nif* D-H IGS region. Primers FGPH 19 and FGPH 273' were constructed for amplification of *nif* H sequences and it amplified the DNA of all the nitrogen fixers. The non-nitrogen fixers, yielded negative results. The *nif* D and *nif* K primers FGPD 807 and FGPK 700' were designed by comparison of 12 sequences including *Klebsiella, Azotobacter* and *Bradyrhizobium* and *Frankia* strain ArI3 (Jamann *et al.*, 1993).

2.9.4. RESTRICTION DIGESTION PATTERNS:

Restriction digestion of a given DNA fragment can give rise to a pattern of fragments which is informative of the location of target sites in the sequence. Mutation may lead to the change in the target sequence. DNA from different members of a genus were analyzed by such a method (Dobritsa S.V., 1985). Jamann *et al.* (1993) used such an approach for typing *Elaeagnus* infective *Frankia* strains.

The extreme sequence conservation in the *rrn* region as well as *nif* region have been used to obtain the restriction fragment length polymorphism. The PCR products were cleaved by ten, 4 base cutting enzymes. Jamann *et al.* (1992) reported homogeneity among the isolates of *Elaeagnus* infectivity group. However, Jamann *et al.* (1993) again studying the RFLP patterns, found different patterns, even within species. This proved the presence of considerable sequence variation in the amplified *nif*D-K fragment. This work was limited to *Elaeagnus-Casuarina* infective *Frankia* strains. Rouvier *et al.* (1996) conducted a similar analysis of a number of *Casuarina* infective strains. Five different groups could be recognised on the basis of the resulting RFLP pattern. It may be noted that Farnandez *et al.* (1989) had reported only a single genomic species for *Casuarina* infective strains based on DNA -DNA hybridization. DNA extracted directly from nodules were amplified using primers specific for *rrn* and *nif* spacer regions. The amplified products were cut using different restriction endonucleases (Bosco *et al.*, 1996). The choice of these endonucleases were due to their capacity to discriminate *Frankia* strains at the genomic species level (Lumini *et al.*, 1992).

2.9.5. GENERATION OF SINGLE SPORE CULTURE IN *FRANKIA* :

Subcultures of *Frankia* have been obtained by inoculating fresh medium with a suspension of fragmented mycelium. Thus single-cell strains of *Frankia* were not available. High germination percentage of spores was reported for *Frankia* strain Ce15 by Tzen and Torrey (1989). Prin *et al.* (1991) obtained the single spore cultures from the *Frankia* strain ORS 140102, isolated from *Hippophae rhamnoides*. 22 single spore isolates showed uniformity with the mother strain. Seminested *nif* D-*nif*K PCR restriction fragment length polymorphism of twelve single spore isolates of *Elaeagnus -Frankia* strain UFI 132715 showed genetic uniformity with the mother strain (Lumini and Bosco, 1996). There were no single spore cultures available for the *Frankia* strains obtained from *Alnus* spp. Purification should be considered a fundamental prerequisite for the molecular characterization of the *Frankia* strains available, including *Alnus* infective strains. Borthakur *et al.* (1996) used calcium alginate beads for immobilizing *Frankia*. Based on this we have developed a novel method for obtaining single spore lines of *Frankia* (Sarma *et al.*, 1998).

MATERIALS
AND
METHODS

Chapter 3

MATERIALS AND METHODS

3.1. ISOLATION AND CONFIRMATION OF *FRANKIA* STRAIN (AnpArP1)

Nodules of *Alnus nepalensis* were collected at Rupa (27° North Latitude and 92.5° East Longitude), Arunachal Pradesh (subtropical region of North East India, 800-2,000 altitude).The nodules were kept on ice and brought to the laboratory for isolation of *Frankia*.

3.1.1 ISOLATION OF *FRANKIA*

3.1.1.1. Surface sterilization:

Nodules were washed thoroughly with water to remove soil and other foreign particles. They were then washed with few drops of detergent and again thoroughly rinsed with distilled water. Nodules were treated with 30% H_2O_2 for 2-3 mins and washed several times with sterile distilled water under a laminar flow cabinet.

3.1.1.2. Isolation of *Frankia* in liquid culture:

Surface sterilized nodules were then kept on a sterile slide and the epidermal layers of nodule lobes were peeled off with two sterile needles. The nodule lobes were washed with distilled water, crushed in 15 mL of DPM medium (see appendix 1 for composition) and incubated in dark at 28±2°C to obtain sufficient biomass. The flasks were gently shaken once a week.

3.2. GENERATION OF SINGLE SPORE CULTURE:

The strains used for generating single spore cultures are listed in Table 3.2. Following steps were devised for generating single spore cultures.

3.2.1. INDUCTION OF SPORULATION :

One month old strains were grown in 15 mL of DPM medium, to which 2 µg/ mL of ampicilline (preparation in Appendix 2) was added (Ganesh, 1993). Culture tubes were incubated at 28$\pm$2°C, without agitation. Cultures were checked from time to time for spore formation.

3.2.2. FILTRATION OF SPORES :

When profuse sporulation was induced, the cultures were filtered through 3 µM glass filter (2.5 cm. diameter, Sigma, USA) to remove hyphae. Then the filtrate was again filtered through 0.9 µM glass filter (2.5 cm diameter, Sigma, USA) to trap the spores that are approximately 1 µM in diameter. The filter was then shaken in fresh DPM. For spore count, 5 µL of this suspension was taken on a haemocytometer and spore density was counted.

3.2.3. DILUTION OF SPORES:

Spore suspensions were diluted using serial dilution method to reach a density of approximately two spores/ drop (each drop being equal to approximately 5 µL of medium) by adding requisite quantity of DPM.

Table 3.2: LIST OF STRAINS USED FOR THE STUDY:

STRAIN	COLLECTION SITE	OBTAINED FROM
AnpST11	Smit, Shillong	Our Laboratory
AnpUS8	Upper Shillong	,,
AnpAG14	AluGudam, Shillong	,,
AnpUS4	Upper Shillong	,,
ACN^{1AG}	Reference strain	Lyon Collection, France
AnpArP1	Arunachal Pradesh	Present study

3.2.4. ENTRAPMENT OF SPORES IN ALGINATE BEADS :

The above dilution was mixed with equal volume of pre sterilized sodium alginate solution (5%w/v) (preparation of sodium alginate in Appendix-2). This mixture was then dropped using sterile 5 mL syringe into 15 mL sterile $CaCl_2$ solution (1% w/v). Beads so formed were kept in referigerator (approximately 10°C) for about 1 hr for hardening and then washed thoroughly with sterile DPM (Sarma *et al.*, 1998).

3.2.5. SELECTION OF BEADS WITH SINGLE SPORES:

Beads were taken individually per cavity block in between two sterile cavity slides. The edges of these slides were sealed with Parafilm® to prevent any contamination. Beads were observed carefully under a phase contrast microscope (Leitz, Switzerland). Beads with single spores were identified and placed individually into separate culture tubes containing 10 mL of DPM and incubated at 28±2°C in dark.

3.3. NODULATION TEST:

Seeds of *Alnus nepalensis* were surface sterilized with 30% H_2O_2 for 10-15 minutes and rinsed with sterile distilled water. The sterilised seeds were kept in dark at 28°C for 1 day and then at room temperature under light (approximately 16,000 Lux). Germinated seeds were then transferred to sterile pouches containing nitrogen free Hoagland solution (1/16 strength) (see Appendix 1). Seed coats were removed to minimize contamination.

In each jar 18-20 seedlings were kept. About 21 days old seedlings were used for inoculation. Seedlings in a single jar were inoculated with a single culture. Both single spore cultures as well as culture of AnpArP1 were tested in this manner. Separate jars for positive control and negative control were also maintained for each inoculant tested.

Seedlings were allowed to grow at room temperature and under normal light intensity. They were checked for pre-nodule and nodule formation at intervals of 18 hr, 24 hr, 36 hr, etc. For confirming the actinorhizal nature of the nodules thus induced, 1 month old seedlings were tested for nitrogenase activity (see section 3.4).

3.4. NITROGENASE ACTIVITY EXPERIMENTS:

Nitrogenase activity was assessed by the acetylene reduction assay using gas chromatography as described by Borthakur *et al.* (1996). For this purpose the gas chromatograph (Hewlett Packard, 4890D) fitted with "Porapak-T" column was used. The temperature of the injection port was maintained at 120°C, the oven temperature was 70°C, and the detector temperature was 175°C. Hydrogen was used as fuel and nitrogen served as carrier gas. The chromatograph was standardised using qualitative test sample of refinery gas supplied by Hewlett Packard. Ethylene produced by reduction of acetylene was measured and used as a parameter for assessing the nitrogenase activity.

3.4.1. ACETYLENE REDUCTION ASSAY OF NODULATED SEEDLINGS :

Seedlings bearing 1 nodule each were taken out carefully from the pouches from the jars. The root portions were cut with sterile blades. They were washed, surface sterilized using 30% hydrogen peroxide and fresh weight of each nodule was taken. They were then placed in sterile vials (7 mL capacity) containing 1 mL of nitrogen free Hoagland solution (Appendix 1). The vials were sealed with sterile air tight stoppers. Using a sterile syringe, 1 mL of air was replaced with 1 mL of acetylene. The vials were incubated at 30°C for 3 hr and gas sampled by injecting into the gas chromatograph as described above.

3.4.2 ACETYLENE REDUCTION ASSAY OF CULTURES :

Two mL of two weeks old cultures were taken in sterile vials (7 mL capacity). The vials were sealed with air tight stoppers. Using a sterile syringe, 1 mL of air from these vials was replaced with 1 mL of acetylene. The vials were incubated at 30°C for 3 hr and reduction of acetylene was measured by injecting the sample (1 mL) into the Gas Chromatograph as described in section 3.4. After the samples were injected, the cultures were transferred to 1.5 mL centrifuge tubes and centrifuged at 5000 rpm (1624 x g) for 2 minutes to pellet out the cultures. The pellet was weighed tp find out the fresh weight.

3.5. ISOLATION OF DNA FROM CULTURES:

When sufficeint biomass developed, the total genomic DNA was isolated using the following protocol (modified from Simonet *et al.*, 1986).

1. The culture was centrifuged at 2,000 rpm (259 x g) and supernatant was discarded.

2. The pellet was gently re-suspended in 1 mL TE buffer (pH 8.0).

3. To this solution, 10 mg/ mL of molecular biology grade Lysozyme (Sigma, USA) and a pinch of Achromopeptidase (Sigma, USA) was added. It was incubated at 25°C for 60 minutes.

4. Then 250 µL of 20% SDS (20 g SDS in 900 mL of water) was added and the mixture was kept at 60°C for 30 minutes and at room temperature for 15 minutes.

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

6. Equal volume of saturated molecular biology grade phenol (Bangalore Genei, India) was added. Then the tubes were gently inverted 5-6 times to ensure proper mixing.

7. They were then centrifuged at 5,000 rpm (1624 x g) and the upper aqueous phase was carefully transferred to a new tube.

8. Equal volume of chloroform (600 µL) was added to each tube, mixed gently as described above and centrifuged at 5,000 rpm for 5 minutes.

9. The aqueous phase was taken in a separate tube. Isopropyl alcohol (360 µL) was added to the tube and kept overnight at room temperature.

10. The tubes were then centrifuged at 20°C for 30 min at 14,000 rpm (12732 x g).

11. The pellet was saved. To this 500 µL of ice cold 70% ethanol was added and the tubes were centrifuged at 13,000 rpm (10978 x g) at 4°C for 30 min using a cooling centrifuge (REMI, India).

12. The pellet was saved and dried in a vacuum desiccator and the DNA was re-suspended in 10 µL of ultra pure water or TE 8 buffer.

3.6. ISOLATION OF DNA FROM NODULES (Modified from Rouvier *et al.*, 1996):

1. Nodule from each seedling was cut from the root portion.

2. Each nodule was then sterilized with H_2O_2 (30% v/v).

3. The nodule was kept in a 1.5 mL eppendorf tube and crushed in 300 µL of warm (30°C) extraction buffer (composition in Appendix 2) with a tissue homogenizer.

4. 10 µL of 20% SDS was added to it and incubated at 65°C for one hour.

5. This was then centrifuged for 10 minutes at 7,000 rpm (3183 x g to remove plant debris, etc. trapped by PVPP.

6. The supernatant was extracted with an equal volume of chloroform/ isoamyl alcohol (24:1, v:v).

7. It was then centrifuged at 13,000 rpm for 5 min. at room temperature.

8. The DNA from the aqueous phase was precipitated with the addition of 2 volumes of cold absolute ethanol and centrifuged at 13000 rpm for 30 min at 4°C.

9. The DNA pellet was washed with 70% ethanol as above and vacuum dried.

10. The DNA was then re-solubilized in 10 μL of ultra pure water or TE buffer (pH 8.0).

3.7. THE AGAROSE GEL ELECTROPHORESIS OF DNA :

The isolated DNA was examined using the gel electrophoresis. For checking the DNA, molecular biology grade agarose was used (Sigma Chem., USA. Hi-Media, India) with different concentrations of agarose, depending up on the size of the DNA fragments to be visualized. The composition of different agarose gels is described in Appendix 2. Appropriate quantity of agarose was added to the requisite volume of 0.5 X TBE buffer, and agarose was solubilised on a heating mantle or inside a microwave oven.

It was poured into the gel casting tray (Bangalore Genei, India) and allowed to polymerize at room temperature.

After polymerization, the gel was kept in the electrophoresis tank, keeping the well end facing the anode. The tank was filled with TBE buffer of same strength as used for the preparation of the gel. It was ensured that the gel was properly submerged in TBE buffer. The electrophoretic assembly was connected to a power supply.

DNA (2 μL) along with 5 μL Type- II loading buffer (Sambrook *et al.*, 1989) were loaded into the wells and current applied at 2 volts/cm.

When the gel had run sufficiently it was stained with ethidium bromide (5 μg/mL) for 30 minutes. The gel was then visualized on a transilluminator (Pharmacia, Sweden / BioRad, USA) and the photographs taken using a 35 mm camera or a Gel Doc 1000 (BioRad, USA).

3.8. AMPLIFICATION OF *FRANKIA* DNA:

For rrn region, amplification was done using primers FGPS989 (Bosco *et al.*, 1992) and FGPS1490′ 16S -23S -ITS region was amplified using primers FGPS989 and FGPL 132′(Ponsonnet and Nesme 1994). The *nif* D-K IGS region was amplified using the primers FGPD807 and FGPK333′(Nalin *et al.*, 1995). Amplification of total 16S rRNA gene was done using primers FGPS6 and FGPS1490′ (Normand *et al.*, 1996). FGPD826′(our lab, unpublished) and FGPH750 (Simonet *et al.*, 1991) were used for the amplification of *nif* H-D IGS region. The list of the primers used is given in Table 3.8.

All activities for preparation of PCR mix were carried out inside a sterile chamber. Primers were gotten synthesized by Bangalore Genei, India. They were diluted to 5 μM working solution which was added to achieve 0.5 μM of each primer per reaction mix. Each dNTP (Bangalore Genei, Cetus, or Boeheringer) was used at a final

Table 3.8: LIST OF THE PRIMERS USED FOR DIFFERENT REGIONS:

Region Amplified	Primer Pairs (Annealing Temp.°C)	Reference	Lab code
nif D-K IGS	FGPD807 FGPK333′ (53)	Jamann *et al.*, 1993 Nalin *et al.*, 1993	MGL1 MGL3
nif D-H IGS	FGPD826′ FGPH750 (55)	Our Lab, Unpublished, Simonet *et al.*, 1991	MGL9 MGL10
Distal 16S rDNA	FGPS989 FGPS1490′ (50)	Bosco *et al.*, 1992 Bosco *et al.*, 1994	MGL4 MGL7
Total 16S rDNA	FGPS1490′ FGPS6 (55)	Bosco *et al.*, 1994, Normand *et al.* 1992	MGL7 MGL8
16S-23S ITS	FGPS989 FGPS132′ (50)	Bosco *et al.*, 1992 Ponsonet *et al.*, 1994	MGL4 MGL5

concentration of 1 µM/ reaction mix of 25µL. Taq polymerase (Cetus, Bangalore Genei, or Boeheringer) was used at a final concentration of 1U/ 25 µL. The appropriate reaction buffer was maintained at 1X concentration.

Different concentrations (1X, 1/10X, 1/100X, 1/1000X) of DNA were tried for the amplification experiment. The total volume of the PCR mix was made up to 25 µL with ultra pure water.

The reaction mix was then placed in a thermal cycler (Gene Amp ® PCR 2400, Perkin Elmer, USA). Amplification conditions were set according to the primer pair in use (Table 3.8.1).

The efficiency of the PCR amplification was checked using agarose gel electrophoresis (section 3.7).

3.9. PCR/ RFLP STUDIES:

3.9.1 CHOICE OF RESTRICTION ENZYMES FOR DIGESTION :

To select different restriction enzymes for digesting the target DNA, computer analysis was done using MacVector® software and Power Macintosh 6100/66 computer. The same software was also used for predicting the expected restriction profiles.

3.9.2. RESTRICTION DIGESTION AND STUDY OF PROFILE :

The amplified DNA was digested using appropriate restriction enzymes. The master mix was prepared using the required amount of DNA, 1X Buffer (according to the recommendation of the suppliers for each enzyme used), and restriction enzymes. The required volume was made upto 20 μL by adding ultra pure water. Each tube containing the mix was then incubated in a water bath at 37°C for 3 hr. (Table 3.9.1). Reaction was stopped by adding 2 μL of gel loading buffer to the mix. The whole mix was then loaded in a 4% agarose gel and the DNA bands obtained were checked and compared with the uncut DNA and the appropriate molecular weight marker as described in section 3.7.

TABLE : 3.9 RESTRICTION MIXES USED FOR DIGESTION OF VARIOUS DNA SEGMENTS

Primer pairs	DNA (µL)	Enzyme (2U)	Buffer (10X, µL)	Reaction Mix (µL)	Reaction Temp(°C)
Distal 16S rDNA	8	*Scr*F1	NE Buffer 4	20	37
16S-23S *rrn* ITS	8	*Nci*1	NE Buffer 2	20	37

RESULTS AND DISCUSSION

Chapter 4

RESULTS AND DISCUSSION

4.1. ISOLATION AND CONFIRMATION OF *FRANKIA* STRAIN:

4.1.1. ISOLATION OF *FRANKIA*:

The nodules collected from Arunachal Pradesh were seen in clumps of nodule lobes (above 2mm in diameter) They were found in the soil at depths between 1-9 cm. Kurdali and Domenach (1991) had also reported similar depths for nodules. Young nodules were whitish-yellow in colour and were of "*Alnus* type". Increase in biomass of *Frankia* in culture was generally obtained after 3-4 weeks. This is according to published reports where it was reported that for normal visible growth period is 4-8 weeks. It may be due to the inability of some *Frankia* cultures to grow in contact with the growth medium (Diem and Dommergues, 1988) and slow doubling rate (Lechevalier and Lechevalier, 1989).

The cultures obtained were whitish in colour. Pin head sized colonies could be seen that changed colour to pale white on subculturing. The colonies settled down to the bottom of the culture tube confirming their micro-aerophillic nature.

4.1.2. CONFIRMATION OF THE IDENTITY OF THE CULTURE OBTAINED FROM THE FIELD COLLECTED NODULES

The culture obtained from the crushed nodule in DPM medium was tested for confirmation of its identity. Alder seedlings were inoculated with the culture (Section 3.3) and it was observed that the culture could nodulate the seedlings within one and a half months. The nodulation percentage was 17.7%.

According to Capellano *et al.* (1987), some *Penicillium* sp also could infect alder and give rise to nodule like outgrowth. These myconodules did not fix nitrogen. Therefore, nodulated alder seedlings were subjected to acetylene reduction assays (section 4.5). All nodulated seedlings tested demonstrated nitrogenase activity, thus discounting the possibility of formation of myconodules.

Simultaneously, the isolate was subjected to DNA amplification tests using *Frankia* genus psecific DNA probes (Bosco *et al.*, 1992). The DNA isolated from the isolate showed positive amplification with the genus specific primer (Fig:4.1.2.A). The DNA also gave positive results for *nif* D-K IGS region (Nalin *et al.*, 1995) (Figs.4.1.2.A, 4.1.2.B) and 16S-23S rRNA ITS region (Bosco *et al.*, 1992 and Ponsonnet *et al.*, 1994) (Fig:4.1.2.C). The change in the size of the amplified product with reducing concentration (Fig:4.1.2.B) may signify the presence of contaminating DNA which competed and interfered with the amplification of target DNA at higher concentrations.

These results confirmed that the isolate obtained from the nodules collected from Arunachal Pradesh was *Frankia*.

4.2. GENERATION OF SINGLE SPORE CULTURES:

Frankia is a slow growing organism (generation time 2-5 days; Diem and Dommergues, 1988), therefore, its growth is overtaken by other contaminating organisms. This poses great problem in obtaining pure cultures. Not only this, while crushing field collected nodules, fragmented hyphae are released into the medium. These fragments then grow into tiny colonies. Thus the cultures obtained tend to have a mixture of genotypes. This makes generation of single spore cultures mandatory.

In the present investigation, the spores trapped in the beads (section 3.2.4) germinated within hours after placing them in the appropriate medium. It was found that about 40-50% spores germinated within 2 to 3 days. The varying time taken for germination by different spores could be because of the differences in their maturity levels. The spores that did not germinate were possibly not viable. Within a week, clear colonies were visible within the beads and in 10-12 days the cultures were seen floating in the liquid medium. Within one month sufficient biomass became available. The beads could be dried and kept in sterile condition and on transfer to liquid cultures they promptly regained their original shape within few hours. The cultures were still viable and the growth rate was same as above. Thus, dried beads could be used for transportation of *Frankia* cultures. Beads can also be used in forest ecosystem as biofertilizers (Borthakur *et al.*, 1997). Single spore (SSP) cultures obtained in this manner from different mother cultures are listed in the table 4.2.

Prin *et al.* (1991) and Lumini and Bosco (1996) also reported methods for generation of single spore cultures. However, they used the agar plate method. It was observed that using the alginate bead method, better and faster growth of *Frankia* could be

Table 4.2.. STRAINS USED FOR GENERATION OF SINGLE SPORE CULTURE

Mother culture	No. of Single spore cultures obtained
AnpST11	3
AnpAG14	3
AnpUS4	3
ACN1^{AG}	3
AnpUS8	5

obtained. Further, the management of contamination was very easy by simply weeding out the contaminated beads.

4.3. CONFIRMATION OF THE IDENTITY OF THE SINGLE SPORE CULTURES:

4.3.1. NODULATION TESTS:

When one month old seedlings were subjected to nodulation tests (section 3.3), root hair deformations were observed within 12 days with all the isolates. After about two weeks pre nodules were observed. Nodules were dark brownish in colour. It is possible that due to the small size of the seedlings, not more than 2-3 nodules were formed per seedling. Negative controls showed stunted growth in comparison to inoculated seedlings and no nodules were formed. This confirmed the ability of isolates to induce nodulation on the host.

4.3.2. NITROGENASE ACTIVITY:

4.3.2.1.Nitrogenase activity of single spore (SSP) cultures:

At least two single spore generated cultures per mother culture were chosen for further investigations. Two weeks old single spore cultures were tested for nitrogenase activity. All of them showed positive results (Fig: 4.3.2.1). Only those cultures were included for further investigations that successfully nodulated the host, thereby demonstrating the generation of single spore purified *Frankia* cultures.

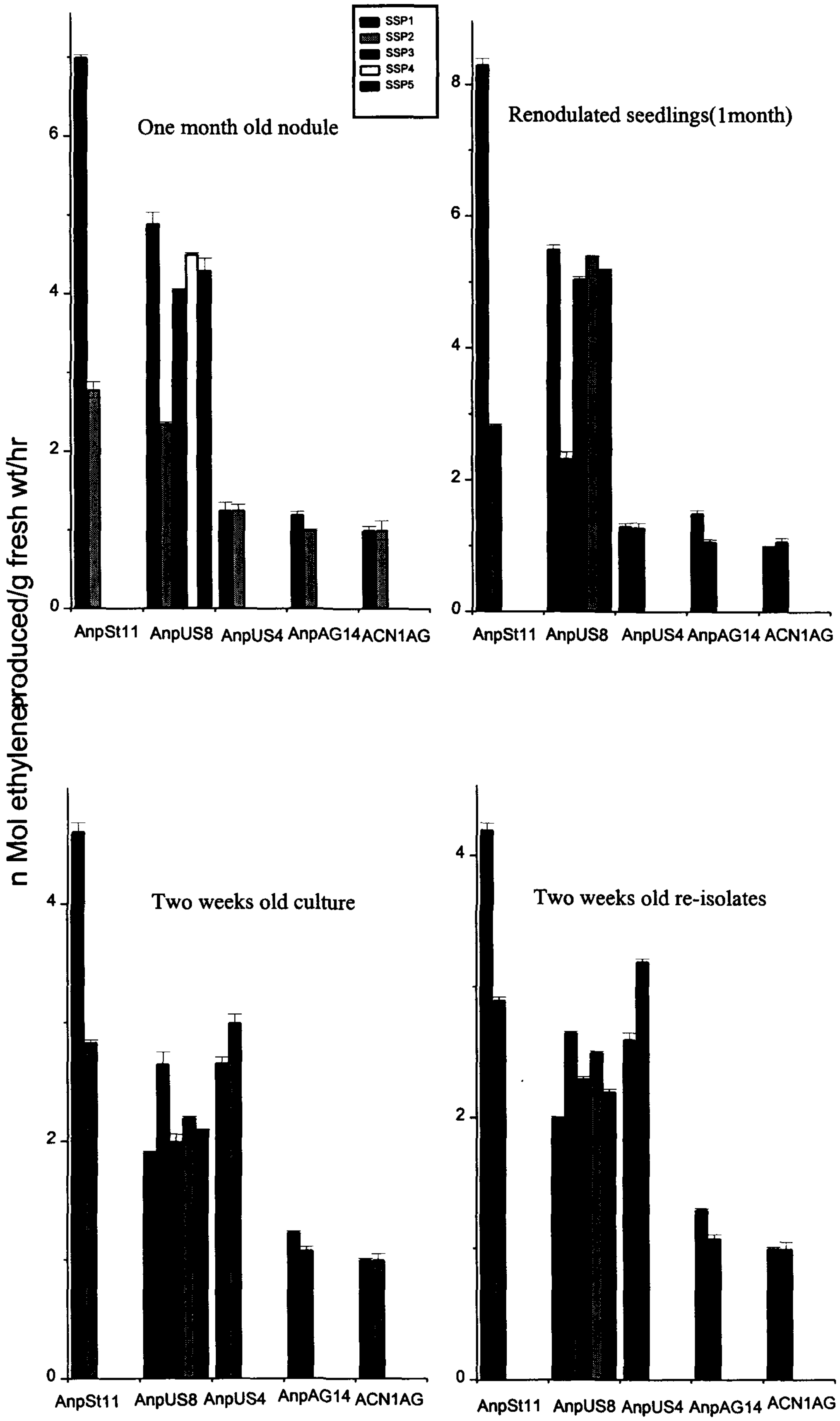

FIG:4.3.2.1. NITROGENASE ACTIVITY OF CULTURES AND SEEDLINGS
(n Mol ethylene produced/g fresh wt/hr)

4.3.2.2. Nitrogenase activity of nodulated seedlings:

Nitrogenase activity was assessed for the seedlings bearing nodules induced by single spore cultures generated as above. One month old cultures were taken for the purpose. A perusal of figure 4.3.2.1 shows that the symbiotic condition had a positive effect on the nitrogenase activity.

4.3.2.3. Nitrogenase activity of re-isolates:

The nodules induced by the single spore cultures were crushed afresh in the DPM. These resulted in to cultures of the `re-isolates'. These cultures were again subjected to the ARA. The pattern and level of nitrogenase activity was found to be similar to that of the corresponding single spore cultures (section 4.3.2.1)(Table4.3.2.1).

4.3.2.4. Nitrogenase activity of re-nodulated seedlings:

The cultures of the above re-isolates were used for re-inoculation of seedlings. They could successfully nodulate the seedlings of alder. The results fulfill Koch's postulate. These nodules were again subjected to ARA. It was observed that these nodules also showed the same pattern and levels of nitrogenase activity as shown by the seedlings in section 4.3.2.2, (Fig: 4.3.2.1).

Therefore it was concluded that the Koch's postulate was met successfully and that the microorganism involved was *Frankia*.

Table:4.3.2.2.: **Acetylene Reduction Assay of cultures and seedlings (nMol. ethylene produced/g fresh wt/hr)**

Samples	Nodulated seedlings	re-nodulated seedlings	Isolates	re-Isolates
AnpST11 ^{SSP1}	7.00	8.30	4.61	4.20
AnpST11^{SSP2}	2.78	2.83	2.83	2.90
AnpUS8^{SSP1}	4.89	5.50	1.91	2.00
AnpUS8^{SSP2}	2.35	2.33	2.65	2.65
AnpUS8^{SSP3}	4.05	5.05	2.00	2.30
AnpUS8^{SSP4}	4.50	5.40	2.20	2.50
AnpUS8^{SSP5}	4.30	5.20	2.10	2.20
AnpUS4^{SSP1}	1.25	1.30	2.66	2.60
AnpUS4^{SSP2}	1.25	1.28	3.00	3.20
AnpAG14^{SSP1}	1.20	1.50	1.23	1.30
AnpAG14^{SSP2}	1.00	1.07	1.08	1.08
ACN1$^{AG/SSP1}$	1.00	1.00	1.00	1.00
ACN1$^{AG/SSP2}$	1.00	1.07	1.00	1.00

4.4. PCR AMPLIFICATION OF *FRANKIA* DNA:

The genomic DNA isolated from various single spore cultures (Fig: 4.4.A1) and corresponding mother cultures were subjected to PCR studies Table 4.4.1).

The amplified product using the two pairs of *Frankia* specific primers MGL4 and MGL7 (Bosco *et al.*, 1992) was approximately 500 bp in size Fig 4.4A2, 4.4.B, 4.4.C, 4.4.H, 4.4.I, 4.4.J, 4.4.K). The amplified product for the primer pairs MGL4 (Bosco *et al.*, 1992) and MGL5 (Ponsonnet and Nesme, 1994) was approximately 830 bp to 1 kb (Fig 4.4.E). The size of the amplified product for *nif* D-K IGS (primers MGL1 and MGL3, Jamann *et al.*, 1993) was approximately 1.3 kb (Fig 4.4.F, 4.4.G, 4.4.I, 4.4.K). The yield of the amplified product of total 16S rRNA gene (MGL7 and MGL8) was of the expected size (1.5 kb, Normand *et al.*, 1996) (Fig 4.4.I, 4.4.K, 4.4.O, 4.4.P). *nif* D-H IGS region got amplified using primers MGL9 (unpublished, our lab) and MGL10 (Simonet *et al.*, 1991)(Fig 4.4.L, 4.4.M, 4.4.N).

From the above studies it can be concluded that the single spore cultures obtained through alginate bead method did nodulate the seedlings of alder and these nodules fixed nitrogen. The genomic DNA got ampified using *Frankia* group specific primers, the *nif*-ITS primers, the *nif*-IGS primers and the 16S rRNA gene primers.

All these results confirmed the identity of the culture as *Frankia*. Hence the single spore cultures of *Frankia* were generated successfully.

TABLE 4.4.1: RESPONSE OF VARIOUS DNA SAMPLES TO DNA AMPLIFICATION FOR DIFFERENT REGIONS USING SPECIFIC PRIMERS

	REGION OF AMPLIFICATION				
	nif-D-K IGS	16S Distal	16S-23S ITS	*nif* D-H IGS	Total 16S rRNA
DNA Used					
AnpArP 1	+	+	+	N.T.	+
ACN[1AG]	+	+	–	+	+
AnpST11	+	+	+	+	+
AnpUS8	+	+	+	N.T.	–
AnpAG14	+	+	+	N.T.	+
AnpUS4	+	+	+	N.T.	+

+ Amplification positive,

- Amplification negative

N.T. Amplification not tried

Figures 4.1.2.A to 4.4.B

Fig: 4.1.2.A- Ampification of AnpArP1 DNA

Using Primers MGL1& MGL3
1-(-ve control), 2- (1X DNA), 3(1/10 X), 4-(1/100 X), 5-(1/1000 X), 6-(1/10 X), L-Lambda DNA, EcoRI/HindIII double digest

Using Primers MGL 4& MGL7
7-(1/10 X), 8-(1 X DNA), 9-(1/1000 X), 10-(1/100 X), 11-(1/100 X), 12-(1/10 X), 13-(-ve control), 14-(1 X),

Fig: 4.1.2.B- Ampification of AnpArP1 DNA

Using Primers MGL1&MGL3

Lane 1-(1 X DNA), 2-(-ve control), 3-(1/10X), 4- (1/100X), 5- (1/1000X)

Fig: 4.1.2.C
Lane1-AnpArP1(1 X DNA), 2-(1/100X), 3-(-ve control)

Fig: 4.4.A1- Genomic DNA
Lane1- AnpUS4^{SSP1},2-ACN1 $^{AG/SSP1}$, 3-AnpST11^{SSP1}, 4-AnpUS8^{SSP1}, 5-AnpUS8^{SSP2}, 6-AnpAG14^{SSP1},7- AnpST11^{SSP1}

Fig: 4.4.A2- Amplification using Primers MGL4&MGL7 (mother culture)

Lane1-AnpUS8(motherculture1),2-AnpUS8(2), 3-AnpST11(1) , AnpUS4(1), 5-AnpAG14(2), 6-AnpUS4(2)

Fig:4.4.B- Amplification using Primers MGL4& MGL7

Lane1-AnpUS8^{SSP1}, 2-AnpUS8^{SSP2}, 3-AnpUS4^{SSP1}, 4-AnpAG14^{SSP1}, 5-(-ve control).

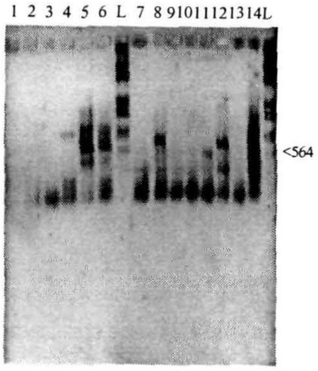

Fig: 4.1.2.A

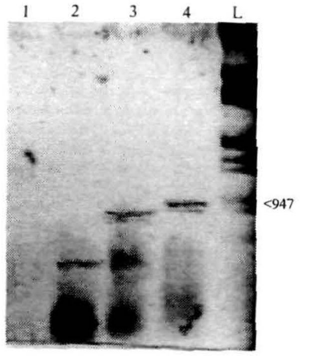

Fig: 4.1.2.B

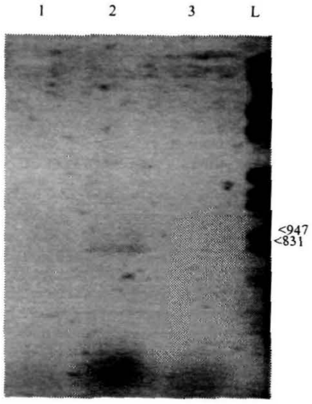

Fig: 4.1.2.C

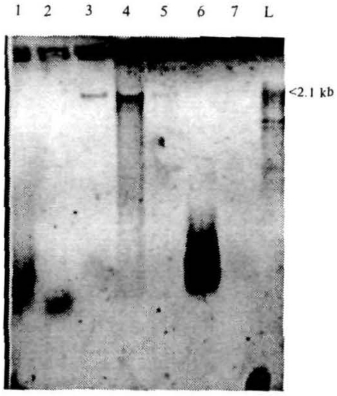

Fig: 4.4.A1

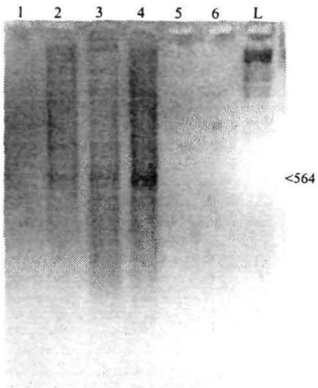

Fig: 4.4.A2

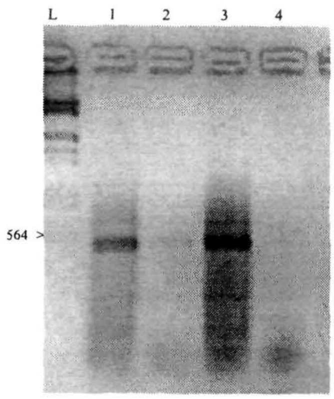

Fig: 4.4.B

Photograph taken with the help of Gel Doc. System, 1000. BioRad, USA

Figures 4.4.C to 4.4.H

Fig: 4.4.C- Amplification using Primers MGL4 & MGL7

Lane1-AnpUS8^{SSP1}, 2-AnpUS8^{SSP2} , 3-AnpST11^{SSP3}, 4-AnpST11^{SSP2}, 5- ACN1$^{AG/SSP1}$, 6-AnpAG14^{SSP2}, 7-(-ve control), 8- (+ve control)

Fig: 4.4.D- Amplification using Primers MGL4&MGL7

Lane1-AnpUS8^{SSP3}, 2-AnpUS8^{SSP4}, 3-AnpUS8^{SSP5}

Fig: 4.4.E- Amplification using Primers MGL4&MGL5

Lane1-AnpST11^{SSP1}, 2- (-ve control), 3-AnpST11^{SSP2}, 4-AnpUS4^{SSP1}, 5-ACN1$^{AG/SSP2}$.

Fig: 4.4.F- Amplification using primers MGL1& MGL3

Lane1-(-ve control), 2-ACN1 $^{AG/SSP1}$, 3-ACN1 $^{AG/SSP2}$ 4-ACN1 $^{AG/SSP3}$,

Fig:4.4.G- Amplification using Primers MGL1&MGL3

Lane1-AnpST11^{SSP1}, 2-(-ve control), 3-AnpUS8^{SSP1}, 4-AnpST11^{SSP2}.5-AnpUS4^{SSP1}.

Fig:4.4.H- Amplification using MGL4&MGL7

Lane1-AnpUS8^{SSP1}, 2-AnpUS8^{SSP2}, 3-AnpUS8^{SSP1} 4-(-ve control), 5-AnpUS8$^{SSP3.}$ 6-AnpUS8^{SSP4} , 7-AnpUS8^{SSP5}

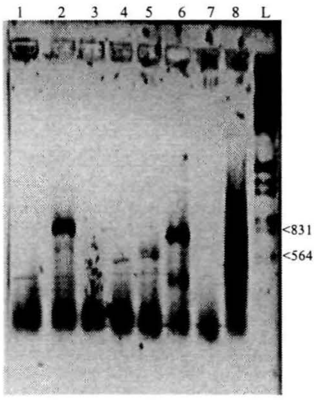

Fig: 4.4.C

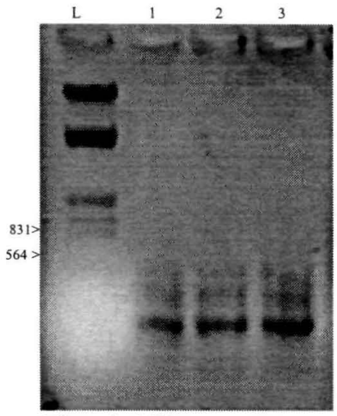

Fig: 4.4.D

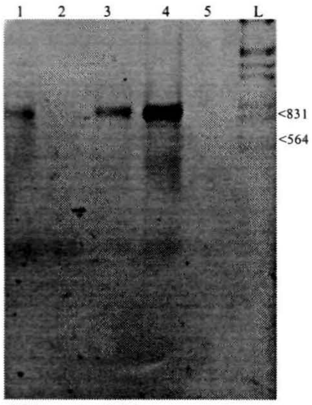

Fig: 4.4.E

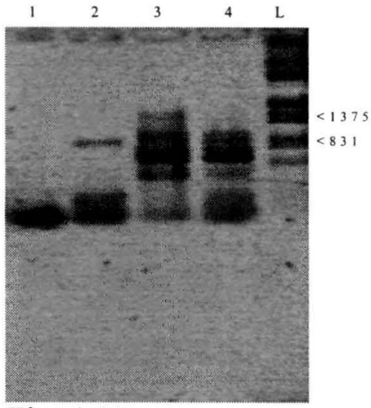

Fig: 4.4.F

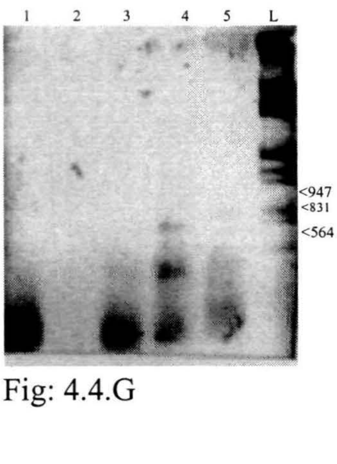

Fig: 4.4.G

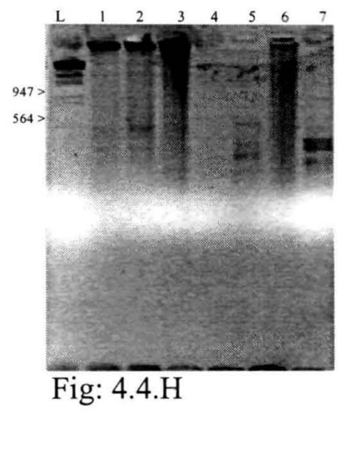

Fig: 4.4.H

Fig:4.4.I- Lane 1-Amplification using Primers MGL7&MGL8 for ACN1 $^{AG/SSP1}$
2-Amplification using Primers MGL4&MGL7 for ACN1 $^{AG/SSP1}$
3- Amplification using Primers MGL4&MGL7 for ACN1$^{AG/SSP2}$
4- Amplification using Primers MGL1&MGL3 for ACN1 $^{AG/SSP1}$

Fig:4.4.J- Amplification using primers MGL4&MGL7
Lane1-AnpAG14^{SSP2}, 2-AnpUS4^{SSP2},
3-ACN1 $^{AG/SSP3}$, 4-ACN1 $^{AG/SSP1}$
5-AnpAG14^{SSP1}, 6-AnpUS4^{SSP1}, 7-(+ve control)

Fig:4.4.K- Lane 1-Amplification using Primers MGL1& MGL3 for AnpUS4^{SSP1}, 2-AnpUS8^{SSP1},
3-Amplification using Primers MGL4& MGL7 for AnpUS4^{SSP2}
4-Amplification using Primers MGL7&MGL8 for AnpUS4^{SSP1}
5- (-ve control) for MGL7&MGL8.
6 -Amplification using Primers MGL7&MGL8 for AnpUS4^{SSP2}

Fig: 4.4.L- Amplification using Primers MGL9&MGL10

Lane1- AnpST11^{SSP1}, 2- AnpST11^{SSP2}, 3-(-ve control).

Fig: 4.4.M- Amplification using Primers MGL9&MGL10

Lane1- AnpUS4^{SSP2}, 2-AnpAG14^{SSP2}, 3-ACN1 $^{AG/SSP1}$,
4-ACN1 $^{AG/SSP2}$.5-(-ve control), 6-AnpUS4^{SSP1}

Fig:4.4N- Amplification using Primers MGL9& MGL10

Lane1-ACN1$^{AG/SSP3}$, 2-(-ve control), 3- AnpUS4^{SSP2},
4-ACN1$^{AG/SSP2}$

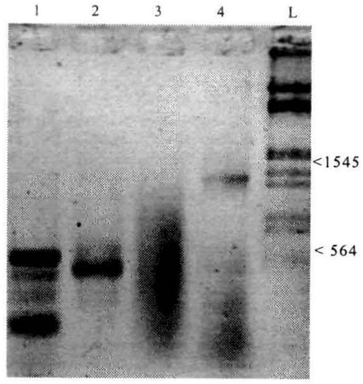

Fig: 4.4.I

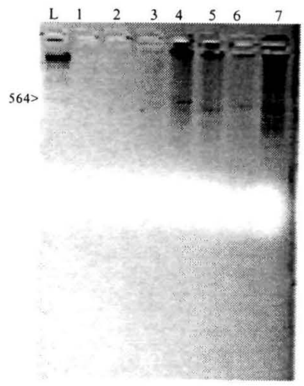

Fig: 4.4.J

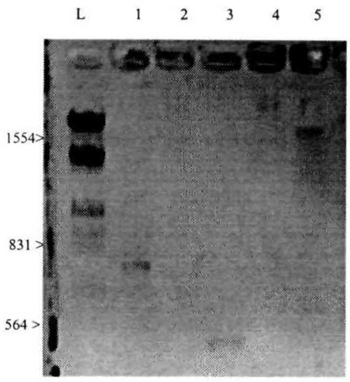

Fig: 4.4.K

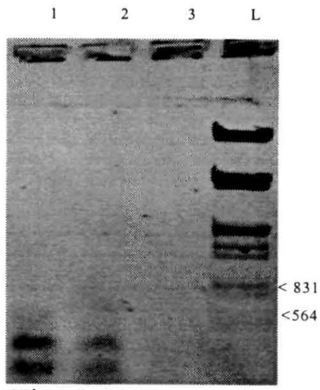

Fig: 4.4.L

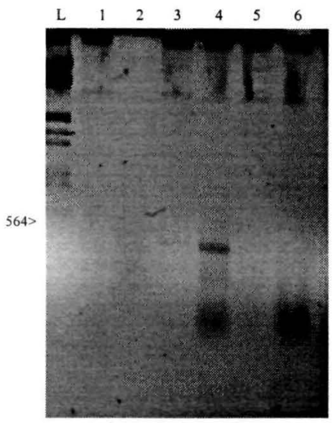

Fig: 4.4.M

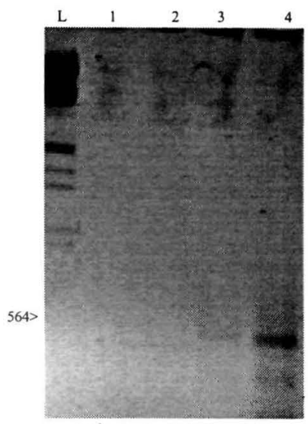

Fig: 4.4.N

Figures 4.4.0 to 4.5.3.D

Fig:4.4.O- Amplification using Primers MGL7&MGL8

Lane1-AnpUS8^{SSP1}, 2-AnpUS8^{SSP2}, 3-AnpST11^{SSP1}, 4-AnpST11^{SSP2}, 5-AnpAG14^{SSP1}.6-AnpAG14^{SSP2}, 7-AnpUS4^{SSP1}, 8-AnpUS4^{SSP2},

Fig:4.4.P- Amplification using Primers MGL7& MGL8.

Lane1-(-ve control), 2-AnpUS4^{SSP3}, 3-ACN1$^{AG/SSP1}$, 4-AnpUS8^{SSP1}, 5- AnpST11^{SSP2}, 6-AnpAG14^{SSP2}, 7-AnpUS8^{SSP2}, 8-AnpAG14^{SSP1}, 9- ACN1$^{AG/SSP3}$, 10-(+ve control).

Fig: 4.5.3.A- Amplified product of 16S rRNA gene Digested Using *ScrF*1

Lane1-AnpUS8^{SSP1} Digested, 2- Undigested DNA
3-AnpST11^{SSP1}Digested, 4- undigested DNA
5-AnpUS8^{SSP2}Digested, 6- undigested DNA

Fig: 4.5.3.B- Amplified product of 16S rRNA gene Digested Using *ScrF*1

Lane 1- AnpUS4 ^{SSP1} Digested, 2- undigested DNA
3-AnpUS8^{SSP2} Digested, 4- undigested

Fig:4.5.3.C- Amplified product of 16S-23S-ITS Digested using *Nci*1

Lane1-ACN1$^{AG/SSP2}$Digested, 2- undigested, 3-(-ve), 4-AnpUS8^{SSP2} Digested, 5- AnpUS8^{SSP2} undigested, L-pBR-322DNA/Hinf digest.

Fig: 4.5.3.D- Amplified product of 16S-23S-ITS digested using N*ci*1

Lane-1-AnpUS8^{SSP1} Digested, 2- undigested, 3-AnpUS4^{SSP1} Digested, 4- undigested, 5-AnpAG14^{SSP2}Digested, 6-AnpAG14 ^{SSP2}undigested.

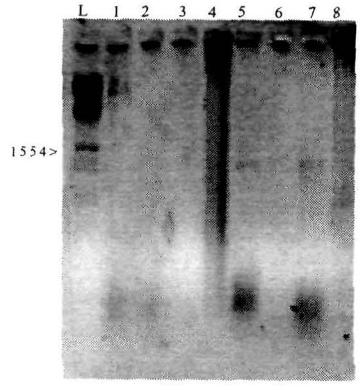

Fig: 4.4.O

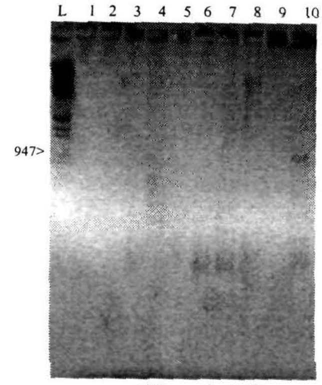

Fig: 4.4.P

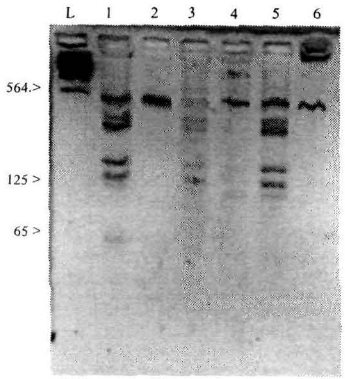

Fig: 4.5.3.A

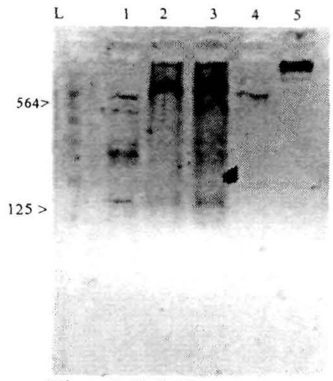

Fig: 4.5.3.B

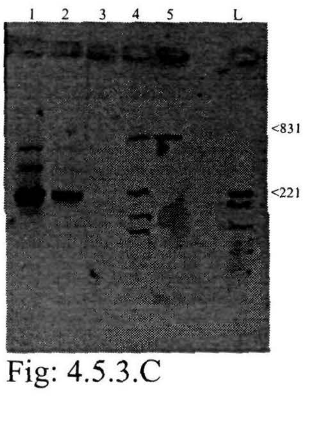

Fig: 4.5.3.C

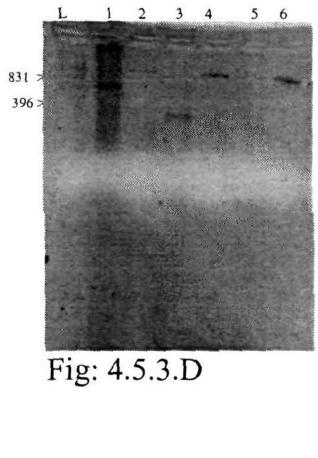

Fig: 4.5.3.D

4.5. CHARACTERISATION OF MOLECULAR DIVERSITY OF THE ISOLATED STRAINS:

4.5.1. DIVERSITY IN RELATION TO NITROGENASE ACTIVITY:

When acetylene reduction assays (ARA) were done for the two weeks old cultures, it was seen that the different single spore cultures (SSP) showed different levels of nitrogenase activity (Table 3.4.2.1). For example AnpST11^{SSP1} produced more ethylene per unit fresh weight of culture than the AnpST11^{SSP2}. AnpST11^{SSP1} had higher nitrogenase activity compared to other cultures as well. This was followed by AnpUS4^{SSP2}. Similarly, AnpUS8^{SSP2} showed higher nitrogenase activity compared to AnpUS8^{SSP1}. All the single spore cultures obtained from one mother strain showed different levels of nitrogenase activity, except those obtained from ACN1^{AG}. Thus, the mother cultures possibly had a mixture of genotypes. In case of the nodulated seedlings also the nitrogenase activity results showed variations (Figure 4.5.1).

Thus, the contention that the mother cultures had mixed genotypes seemed to be right. This also justified the need to generate single spore cultures.

As regards the absence of variability among single spore cultures obtained from ACN1^{AG}, it seems that the dominant genotype has survived the several cycles of subculturing that this strain must have undergone since its isolation. Other variants, if any, were probably less competetive for the conditions of culture and were, therefore, eliminated.

4.5.2. VARIATION IN RELATION TO PCR AMPLIFICATION:

4.5.2.1. Variation in respect of amplified fragment length profile (AFLP):

4.5.2.1.1. AFL Profile Using primers MGL4 and MGL7 :

Fragments of different sizes were observed when DNA of different single spore cultures were amplified using these two primers. While $AnpUS8^{SSP1}$ and $AnpUS8^{SSP2}$ showed single bands on amplification (Figure 4.4.H), $AnpUS8^{SSP3}$, $AnpUS8^{SSP4}$ and $AnpUS8^{SSP5}$ showed multiple bands (Figure 4.4.D). On computer analysis of unpublished $ACN1^{AG}$ DNA sequence, it was found that primer MGL4 could have annealed at two more poisitions along the target DNA (Table 4.5.2.1.1.). Such annealing would have occurred with up to six mismatches between the primer and target DNA. Thus, the primer could have annealed at three positions giving rise to amplification products of sizes 500 bps (expected), 400 bps and 200 bps approximately (Figure 4.4.D). However, since these bands did not dissappear on raising the annealing temperature, the number of mismatches were actually fewer than the computer prediction. The samples that did not yield multiple bands obviously had more mismatches at these positions.

Similar computer analysis for primer MGL7 did not yield any alternate sites for its annealing. Therefore, the multiple bands obtained were due to multiple annealing sites for MGL4.

Table 4.5.2.1.1: Computer simulated multiple annealing sites for primer MGL4 in the distal part of 16S rRNA gene sequence of ACN1^{AG}

#Res. Pos.	Bases Matched	Strand	Subseq Found
989	16/16	+	GGGGTCCGTAAGGGTC
1120	10/16	+	GGaGaCtGccgGGGTC
1319	10/16	-	ccaGgaCGTAAGGGgC
1590	10/16	+	GGtcTCaGTtcGGaTC

The mismatches have been shown in lower case letters.

Figures 4.4.E to 4.5.3.G

Fig:4.5.3.E- Amplified product of 16S-23S-ITS Digested using *Nci1*

Lane-1- ACN1$^{AG/SSP2}$ Digested, 2- undigested, 3-AnpUS4^{SSP1} Digested, 4- undigested

Fig: 4.5.3.F- Amplified product of 16S-23S-ITS digested using *Nci1*

Lane1- AnpAG14^{SSP1} Digested, 2- undigested, 3-AnpUS8^{SSP1} Digested, 4- AnpUS8^{SSP1} undigested,

Fig: 4.5.3.G- Amplified product of 16S-23S-ITS digested using *Nci1*

1- AnpUS8^{SSP1} Digested, 2- undigested, 3-ACN1$^{AG/SSP2}$ Digested, 4- ACN1$^{AG/SSP2}$ undigested.

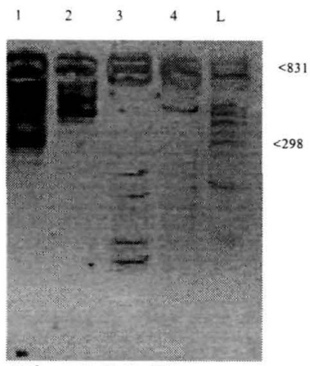

Fig: 4.5.3.E

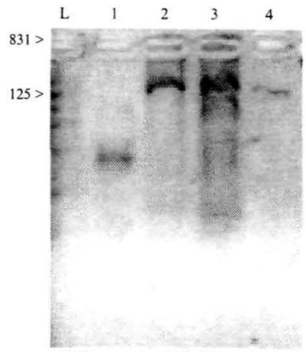

Fig: 4.5.3.F

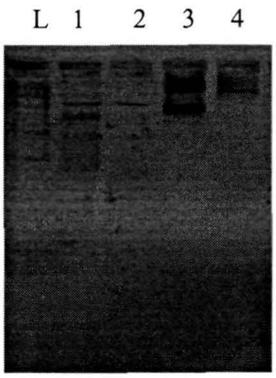

Fig: 4.5.3.G

4.5.2.1.2. AFL profile using primers MGL1 and MGL3 :

PCR products of *nif* D-K IGS for ACN1$^{AG/SSP2}$ and ACN1$^{AG/SSP3}$ showed multiple bands (Figure 4.4.F, 4.4.G, 4.4.I). Jamann *et al.* (1993) also had reported generation of multiple bands when these primers were used by them. On computer analysis of ArI3 sequence, it was found that *nif* D primer could anneal with varying degree of mismatches to the target DNA at least at three sites, giving rise to bands of approximately 830, 600 and 1300 base pairs. The bands of size bigger than 1.3 kb obtained by us could have arisen because of alternate site annealing of primer MGL3. However, these bands got eliminated when annealing temperature was increased.

4.5.3. DIVERSITY IN RELATION TO PCR-RFLP STUDIES:

PCR-RFLP has been found to permit rapid bacterial identification (Osborn *et al.*, 1993). The amplified DNA obtained from these cultures for different regions as above, were digested using different enzymes. Restriction enzymes *ScrF*1 and *Nci*1 were used for *rrn* and *nif* ITS regions respectively. Each enzyme gave two to four different restriction patterns.

When the amplified DNA of 16S-23S ITS were digested with *Nci*1, different patterns were observed (Figures 4.5.3.C, 4.5.3.D; 4.5.3.E, 4.5.3.F, 4.5.3.G). In majority of the cases three similar bands were observed. In case of AnpUS8^{SSP1}, however, there were four bands (Figure 4.5.3.F). The published DNA sequence of *Casuarina* compatible strain ORS020606 was subjected to computer analysis using MacVector software. Computer simulated digestion of the

Table 4.5.3.: Computer simulated digestion of published sequence of ORS020606 by enzyme *Nci1*.

Fragment Size	From : To
284	1494:2141
223	1130:1493
189	3724:4790
174	2846:3723
140	989:1129
56	2142:2845

same region using *NciI* predicted six fragments (Table 4.5.3). While four predicted fragments were similar to that of AnpUS8^{SSP1}, only three matched with the fragments obtained in other cases (Figure 4.5.3.F, Lane3). Interestingly, AnpUS8^{SSP2} had a pattern similar to other single spore cultures (Figure 4.5.3.C, Lane4) .

In case of AnpUS4^{SSP1} the digested bands showed the pattern expected of alder *Frankia*. However, in case of AnpAG14^{SSP1} a smaller fragment was observed. It may be pointed out that the size of the amplified product itself was smaller compared to others (Figure 4.5.3.F, Lane 1). Therefore, the smaller fragment generated on digestion could be a product of smaller ITS region in this case.

When the amplified DNA for the distal part of the 16S rRNA gene were digested with *ScrF1*, all the alder strains showed similar patterns except AnpUS8^{SSP1}, where an extra band was present (Fig 4.5.3.A). This extra band was also predicted in a computer simulated digestion of *Casuarina* compatible strain ORS020606.

Thus unique PCR-RFLP profile was found for AnpUS8^{SSP1}. Further testing using other enzymes may yield similar results for other single spore purified strains. There is a need to continue this work till specific pattern for all purified strains are developed.

CONCLUSION

Chapter 5

CONCLUSION

The following conclusions can be drawn from the studies carried out:

1. The isolate AnpArP1, obtained from nodules collected from Arunachal Pradesh, was *Frankia,* since it nodulated the *Alnus nepalensis* seedlings and the nodulated seedlings too fixed nitrogen.

2. A novel method for genetic purification of *Frankia* cultures was devised. The single spore cultures obtained through this method fulfilled Koch's postulate by renodulating the alder seedlings. The nodulated seedlings also showed nitrogenase activity.

   The DNA of the single spore purified cultures got amplified when genus specific primers were used. Positive amplification was also obtained for *nif*D-K IGS, *nif*D-H IGS, total 16S rRNA gene and 16S-23S ITS. All these amplification studies confirmed the identity of the isolates to be *Frankia*.

3. PCR-RFLP studies as well as nitrogenase activity studies confirmed the presence of variability among single spore cultures obtained from same mother culture. $AnpST11^{SSP1}$ showed higher nitrogenase activity than the other isolates.

4. AnpUS8^{SSP1} showed a PCR-RFL profile very different from other alder strains studied. This can thus be used as a signature for AnpUS8^{SSP1}.

APPENDICES

APPENDICES

APPENDIX 1:

Composition of Culture Media

DPM (N_2 FREE) (Modified from, Baker and O' Keefe, 1984)

Constituents	Concentrated stock solution	Working Solution(Per Liter)
(A) Macro nutrients	10X	
KH_2PO_4	10.0 g	7348mM
$MgSO_4$	1.0 g	0899mM
$CaCl_2$ $2H_2O$	0.1 g	0088mM
$C_3H_5O_2Na$	12.0 g	12490mM
(B) Micro nutrients	1000X	
H_3BO_3	2.860 g	46.3mM
$MnCl_2$ $4H_2O$	1.810 g	9.1mM
$ZnSO_4$ $7H_2O$	0.220 g	0.8mM
$CuSO_4$ $5H_2O$	0.080 g	0.3mM
$CoCl_2$ $7H_2O$	0.025 g	0.1mM
(C) Iron solution	1000X	
$C_{10}H_{12}N_2NaFeO_8$	0.004 g	0 .00001mM

HOAGLANDS MEDIUM (Modified from Hoagland and Arnon., 1950)

Constituents	Stock solution	Working solution
(A) Macronutrients	10X	
KH_2PO_4	1.36 g	999mM
$MgSO_4$	3.38 g	3039mM
(B) Micro nutrients	1000X	
H_3BO_3	2.860 g	436.3mM
$MnCl_2$ $4H_2O$	1.810 g	9.1mM
$ZnSO_4$ $7H_2O$	0.220 g	0.8mM
$CuSO_4$ $5H_2O$	0.080 g	0.3mM
Na_2MoO_4	0.025 g	0.1mM
$CoCl_2$ $7H_2O$	0.025 g	0.1mM
(C) Iron solution	0.004 g	0.00001mM

APPENDIX 2:

DNA extraction buffer: (1X Concentration)

100mM molecular biology grade Tris base (pH 8.0)

20mM EDTA (pH 8.0)

1.4 M NaCl

2% (W/V) CTAB

1% (W/V) PVP

EDTA was dissolved in 200 mL of water and the pH was adjusted to 8.0. Then Tris base, CTAB , NaCl and PVP were added to it. The volume was made upto 1000mL. The extraction buffer was autoclaved.

DNA Loading Buffer (Type II): (Sambrook *et al.*, 1989)

6X Buffer

0.25% Bromophenol blue

0.25% Xylene Cyanol FF

15% Ficoll (Type 400; Pharmacia) in water.

Ficoll was dissolved in 50 mL of water . When ficoll was completely dissolved, other chemicals were added to it. The final volume was made upto 100mL. The whole mix was then filter sterilized with 0.2mM cellulose nitrate filter (Sartorius, Germany).

Electrophoresis buffer (TBE) :

5X stock solution (Per liter)	Working Solution (0.5X)
Tris Base -54 g	0.045 M
Boric acid 27.5 g	0.001 M
EDTA (0.5 M)	20 mL

TRIS EDTA buffer (TE pH 8.)

10 mM Tris HCl

1mM EDTA (pH8.0)

EDTA was dissolved in minimum quantity of water and the pH was adjusted to 8.0 then Tris HCl of required quantity was added to it.

PREPARATION OF ANTIBIOTICS :

Ampicillin: Stock of 25mg/ml of the sodium salt of ampicillin was prepared on water. The solution was sterilized by filtration and then stpored in aliqouts at -20°C.

PREPARATION OF SODIUM ALGINATE BEADS :

5g of sodium alginate was dissolved in 100 mL of water and autoclaved. The medium containing filtered spores were mixed with sterile sodium alginate (equal volume). The mix was dropped using sterile 5mL syringe, into 15 mL of sterile 1% (w/v) $CaCl_2$ solution. The beads so formed were kept at approximately 10°C for hardening. After one hour or so the beads were taken out and washed with DPM medium Thoroughly.

PREPARATION OF AGAROSE GEL:

Molecular biology grade (Hi-Media-India, Sigma-USA) was weighed and dissolved in 0.5X TBE buffer by keeping them on a heating mentel or inside a microwave oven. Different concentration and different size of agarose gels were used for the study, there composition is given below-

Tray Size	TBE Used	Agarose			
		0.8%	2%	4%	4.5%
7.5x8mm	25mL	0.2g	0.5g	1.0g	1.12g
8x14mm	40mL	0.32g	0.8g	1.6g	1.18g
14x14mm	100mL	0.8g	2.0g	4.0g	4.5g

REFERENCES

Chapter 6

REFERENCES

An C.S., Wills J.H., Riggsby W.S. and Mullin B.C. (1983). Deoxyribonucleic acid base composition of twelve *Frankia* isolates. Can. J. Bot., **61:** 2859-2862.

An C.S., Riggsby W.S. and Mullin B.C. (1985). Relationships of *Frankia* isolates based on deoxyribonucleic acid homology studies. Int. J. Syst. Bacteriol., **35:** 140-146.

Baker D.D. (1982). A cumulative listing of isolated frankiae, the symbiotic nitrogen-fixing actinomycetes. Actinomycetales, **17:** 35-42.

Baker D. and O'Keefe D. (1984). A modified sucrose fractionation procedure for the isolation of frankiae from actinorhizal root nodules and soil samples. Plant and Soil, **78:** 23-28.

Baker D. and Schwintzer C.R. (1990). Introduction . *In* The Biology of *Frankia* and actinorhizal plants, C.R. Schwintzer and J.D. Tjepkema (eds). Academic press Inc. , California, USA, pp. 1-13.

Becking J.H. (1970). Frankiaceae fam. nov. (Actinoycetales) with one new combination and six species of the genus *Frankia* Brunchorst 1886, 1974. Int. J. Syst. Bacteriol., **20:** 201-220.

Benson D.R. and Silvester W.B. (1993). Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. Microbial Rev., **57**: 293-319.

Bergersen F.J. and Postgate J.R. (1987). A Century of Nitrogen Fixation Research: Present Status and Future Prospects., The royal Society, London. .

Berry A.M. and Sunell L.A. (1990). The infection process. *In* The Biology of *Frankia* and Actinorhizal Plants, C.R. Schwintzer and J.D. Tjepkema (eds). Academic Press Inc. , California, USA, pp. 61-81.

Bloom R.A., Lechevalier M.P. and. Robert T. (1989). Physiological, chemical, morphological and plant-infectivity characteristics of *Frankia* isolates from *Myrica pensylvanica* : Correlation to DNA restriction patterns. Appl. Environ. Microbiol., **55**: 2161-2166.

Borthakur M., Sen A. and Misra A.K. (1997). Exogenus nitrogen sources do not drastically reduce nitrogenase activity in polymer entrapped *Frankia*. Ind. J. Exp. Biol., **35**: 173-175.

Bosco M., Fernandez M.P., Simonet P., Materassi R. and Normand P. (1992). Evidence that some *Frankia* sp. strains are able to cross boundaries between *Alnus* and *Elaeagnus* host specificity groups. Appl. Environ. Microbiol., **58**: 1569-1576.

Bosco M., Lumini E. and Normand P. (1996). PCR-RFLP direct fingerprinting of uncultured *Frankia* microsymbiont from

Dryas drummondii nodules. Annali di Microbiologia ed Enzimologia., **46:** 115-123.

Bousingault J. (1838). Recherches chimiques sur la vegetation, etc. Annls. Chim. Phys. ser.,2. **67: 1-54.

*Brunchorst J. (1885). Über die knollchen an den leguminosenwurzeln. Ber. Dtsc. Bot. Ges., **3**: 241-257.

Burggraaf A.J.P., Quispel A., Tak T. and Valstar J. (1981). Methods of isolation and cultivation of *Frankia* species from actinorhizas. Plant and Soil, **61:** 157-168.

*Burggraff A.J.P. and Valstar J. (1984). Heterogenety within *Frankia* sp. LDAgp1 studied among clones and reisolates. Plant and Soil, **78**: 29-43

Callaham D., Tredici P.D. and Torrey J.G. (1978). Isolation and cultivation *in vitro* of the actinomycete causing root nodulation in *Comptonia*. Science, **199**: 899-902.

Capellano A., Deqatre B., Valla G. and Moiroud A. (1987). Root nodules formation by *Penicillium* sp. on *Alnus glutinosa* and *Alnus incana*. Plant and Soil,**104:** 45-50.

Chapin III F.S., Moilanen L. and Kielland K. (1993). Preferential use of organic nitrogen for growth by a non-mycorhizal arctic sedge. Nature, **361**: 150-154.

---

*- Original not seen please see Schwintzer and Tjepkema (1990).

**- Original not seen please see Bergersen and Postgate (1987).

Dawson J.O. (1990). Interactions among actinorhizal plant species. Interactios among actinorhizal associated plant species. *In*, The Biology of *Frankia* and Actinorhizal Plants, C.R. Schwintzer and J.D. Tjepkema (eds). Academic press Inc. California, USA, pp. 299-316.

Diem H.G., Gauthier D. and Dommergues Y.R. (1983). An effective strain of *Frankia* from *Casuarina* sp. Can. J. Bot., **61**: 2815-2821.

Diem H.G. and Dommergues Y.R. (1988). Isolation, characterization and cultivation of *Frankia*. *In* "Biological Nitrogen Fixation, Recent Developments", N.S. Subbha Rao (ed.). Gordon and Breach Science Publishers, New York. pp. 227-254.

Diem H.G. and Dommergues Y.R. (1990). Current and potential uses and management of Casuarinaceae in the tropics and subtropics. *In* The Biology of *Frankia* and Actinorhizal Plants, C R. Schwintzer and J.D. Tjepkema (eds). Academic Press Inc., California, USA. pp. 317-338.

Diem H.G., Khalifa K.B., Neyra M. and Dommergues Y.R. (1989). Recent advances in the inoculant technology with special emphasis on plant symbiotic microorganisms. *In* Proceedings of Workshop on Advance Technologies for Increased Agricultural Production, (U. Leone, G. Rialdi and R. Vanore, eds). CNR-USG Italy, Santa Mergherita Ligure. pp. 196-210.

Dobritsa S.V. (1985). Restriction analysis of the *Frankia* spp. genome. FEMS Microbiol. Lett., **29**: 123-128.

Dobritsa V.S. and Stupar O.S. (1989). Genetic heterogeneity among *Frankia* isolates from root nodules of individual actinorhizal plants. FEMS Microbiol.Letters, **58:** 287-292.

Fernandez M.P., Meugnier H., Grimont P.A.D. and Bardin R. (1989). Deoxirebonucleic acid relatedness among members of the genus *Frankia*. Int. J. of Syst. Bacteroil., **39.**424-429.

*Frank B. (1987). Sind die wurzelanschwellungen der Erlen und Elaeagnaceen Pilzgallen? Ber. Dtsch. Bot. Ges., **5:** 50-58.

Ganesh G. (1993). Study of genetic diversity of *Frankia alni* strains isolated from *Alnus nepalensis* root nodules found in Meghalaya. Ph.D. Thesis. NEHU, Shillong, India.

Ganesh G., Misra A.K., Chapelon C. and Normand P. (1994). Morphological and molecular characterization of *Frankia* sp. isolates from nodules of *Alnus nepalensis* Don. Arch. Microbiol., **161**: 152-155.

*Gravis A. (1879). Observations anatomiques sur les excroissances des recines de l'aulne. Bull. Soc. R. Bot. Belg., **18:** 369-386.

**Hellrigel H. and Wilfarth H. (1888). Untersuchungen uber die Stickstoffernahrung der Gramineen und Leguminosen. Beil. Z. Ver. dt. ZuckInd., (234 pages).

Hafeez F., Akkermans A.D. and Chaudhary A.H. (1984). Morphology, physiology and infectivity of two *Frankia* isolates An1 and An2 from root nodules of *Alnus nitida* . Plant and Soil, **78**: 45-59.

*Hoagland D.R., and Arnon D.R. (1950). The water culture method for growing plants without soil. Calif. Agri. Exptl. Stn. Circ., 347

Hahn D., Dorsch M., Stakebrandt E. and Akkermans A.D.L. (1989). Synthetic oligonucleotide probes for identification of *Frankia* strains. Plants and Soil, **118** : 211-219.

Hahn D., Kester R., Starremburg M.J.C. and Akkermans A.D.L. (1990). Extraction of ribosomal RNA from soil for detection of *Frankia* with oligonuceotide probes. Arch. Microbiol., **154**: 329-335.

*von Jäger G. (1860). Uber eine Krankhafte Veranderung der Bluthenorgane der Weintraube. Flora (jena)., **18**: 49-51.

Jamann S., Fernandez M.P. and Moiroud A. 1992. Genetic diversity of Elaeagnaceae-infective *Frankia* strains isolated from various soils. Acta Oecologica, **13**: 395-405.

Jamann S., Fernandez M.P. and Normand P. (1993). Typing method for N_2-fixing bacteria based on PCR-RFLP- application to the characterization of *Frankia* strains. Mol. Ecol., **2:** 17-26.

Kurdali F. and Domenach A.M. (1991). Evaluation of nodulation capacity in *Alnus glutinosa* seedlings grown in soil sampled from various depths. Acta Oecologica, **12:** 397.

*Lalonde M. and Calvert H.E. (1979). Production of *Frankia* hyphae and spores as an infective inoculant for *Alnus* species. *In* "Symbiotic Nitrogen Fixation in the management on temperate forests",(J. C. Gordon, C.T. Wheeler and D. A. Perry,eds.). pp. 95-110. For. Res. Lab. Oregon State Univ. Corvallis, Origon.

Lalonde M., Simon L., Bousquet J. and Seguin A. (1988). Advances in the taxonomy of *Frankia* :Recognition of species alni and elaeagni and novel sub species *pommerii* and *vandijkii*. *In*, "Nitrogen Fixation : Hundred Years After,(H. Pothe, F.J. de Bruijn, and W.E. Newton, eds). pp. 671-680.

*Lechevalier M.P. (1986). Nitrogen-fixing actinomycetes of the genus *Frankia*. *In*, Perspectives in Microbial Ecology, F. Megusar and M. Gantar (ed.), Slovene Society for Microbialogy, Ljubljana, Yugoslavia, pp. 253-256.

Lechevalier M.P. and Lechevalier H.A. (1989). Genus *Frankia* Brunchorst 1886, 174AL, In Bergey's Manual of Systematic Bacteriology, A.Deitz and D.W.Thayer (eds.), 4: 2410-2417.

Lechevalier M.P. and Lechevalier H.A. (1990). Systematics, isolation and culture of *Frankia*. *In* The Biology of *Frankia* and Actinorhizal Plants, C. R. Schwintzer and J.D. Tjepkema (eds). Academic Press Inc. California, USA. pp. 35-60.

Lechevalier M.P. (1994). Taxonomy of the genus *Frankia* (Actinomycetales). Minireview. Int. J. of Syst. Bot., **44**: 1-8.

Lumini E., Bosco M., Favilli F. and Wheeler C.T. (1992). Production of spores and nodules of *A. glutinosa* inoculated with non-host *Frankia* strains. Acta Oecologica, **13:** 506-507.

Lumini E. and Bosco M. (1996). PCR-restriction fragment length polymorphisms identification and host range of single spore isolates of flexible *Frankia sp.* strain UFI132715. Appl. Environ. Microbiol., **62:** 3026-3029.

Meesters T.M., van Genesen S.Th. and Akkermans A.D.L. (1985). Growth, acetylene reduction activity and localization of nitrogenase in relation to vesicle formation in *Frankia* strains Cc1.17 and Cp1.2. Arch. Microbiol., **143**: 137-142.

*Meyen J. (1829). Über das hervorwachsen parasitischer Gebilde aus den Wurzeln anderer Pflanzen. Flora (jena)., **12:** 49-64.

*Möller H. (1885). *Plasmodiophora alni.* Ber. Dtsch. Bot. Ges., **3**: 102-105.

Nalin R., Domenach A.M. and Normand P. (1995). Molecular structure of the *Frankia* spp. *nif* D-K-intergenic spacer and design of *Frankia* genus compatible primer. Molecular Ecology, **4:** 483-491.

Nazaret S., Cournoyer B., Normand P. and Simonet P. (1991). Phylogenetic relationships among *Frankia* genomic species determined by use of amplified 16S rDNA sequences. J. Bacteriol., **173**: 4072-4078.

Newcomb W. and Pankhurst C.E. (1982). Fine structure of actinorhizal root nodule of *Coriaria arborea* (Coriariacea). N. Z. J. Bot., **20:** 93-108.

Normand P., and Lalonde M. (1982). Evaluation of *Frankia* strains isolated from provenances of two *Alnus* species. Can. J. Microbiol., **29**: 1133-1142.

Normand P., Cournoyer B., Simonet P. and Nazaret S. (1992-a). Analysis of a ribosomal RNA operon in the actinomycete *Frankia*. Gene, **111:** 119-124.

Normand P., Gouy M. and Cournoyer B. (1992-b). Nucleotide sequence of *nif*D from *Frankia alni* strain ArI3: Phylogenetic interference. Mol. Biol. Evol., **20**: 495-506.

Normand P., Orso S., Cournoyer B., Jeannin P., Chapelon C., Dawson J., Evtushenko L. and Misra A.K. (1996). Molecular phylogeny of the genus *Frankia* and related genera and emendation of the family Frankiaceae. Int. J. Syst. Bacteriol., **46**: 1-9.

*Oersted A.S. (1865). Smaa biologiske og morphologiske bidrag.6. *Hippopha' rhamnoides*. Bot. Tidsskr. Ser 3., **1**: 108-110.

Osborn A.M., Bruce K.D., Strike P. and Ritchie D.A. (1993). Polymerase Chain Reaction-Restriction fragment Length Polymorphism analysis shows divergence among mer determinants from Gram-negative soil bacteria indistinguishable by DNA-DNA hybridization. Appl. Env. Microbiol., **59:** 4024-4030.

Ponsonnet C., and Nesme X. (1994). Identification of *Agrobacterium* strains by PCR-RFLP analysis of pTi and chromosomal regions. Arch. Microbiol., **161**: 300-309.

Prin Y., Neyra M. and Diem H.G. (1990). Estimation of *Frankia* growth using Bradforth protein and INT reduction activity estimations: application to inoculum standardization. FEMS. Microbiol. Letters, **69:** 91-96.

Prin Y., Maggia L., Picard B., Diem H.G. and Goullet Ph. (1991). Electrophoretic comparison of enzymes from 22 single - spore cultures obtained from *Frankia* strain ORS140102. FEMS. Microbiol. Letters, **77:** 223-228.

*Quispel A. (1954). Symbiotic nitrogen fixation in non-leguminous plants. 1. Preliminary experiments on the root-nodule symbiosis of *Alnus glutinosa* (L.) Gaertn. Acta. Bot. Ges., **72:** 138-150.

Reddell P. and Bowen G.D. (1985). Do single nodule of Casurinaceae contain more than one strain? Plant and Soil, **93:** 293-298.

Rouvier C., Prin Y, Reddell P., Normand P. and Simonet P. (1996). Genetic diversity among *Frankia* strains nodulating members of the family Casuarinaceae in Australia revealed by PCR and restriction fragment length polymorphism analysis with crushed root nodules. Appl Environmental. Microbiol., **62:** 979-985.

Sambrook J., Fritsch E.F. and Maniatis T. (1989). Molecular cloning a laboratory manual. (2nd ed), CSH Lab. Press. USA.

Sarma G., Sen A., Varghese R and Misra A.K. (1998). A novel technique for isolation of *Frankia* and generation of single spore cultures. Can. J. Microbiol., **44:** 490-492.

*Shacht H. (1853). Beitrag zur Entwicklungsgeschichte der Wurzel. Flora (jena)., **36**: 257-266.

Schwintzer C.R. (1990). Spore -positive and Spore-negative nodules. *In* The Biology of *Frankia* and Actinorhizal Plants, C. R. Schwintzer and J.D. Tjepkema (eds). Academic Press Inc. California, USA. pp. 178-199.

Sellstedt A. and Kerstin H.D. (1984). Growth, nitrogen fixation and relative efficiency of nitrogenase in *Alnus incana* grown in different cultivation systems. Plant and Soil, **78**: 147-158.

*Shibata K. (1992). Cytologische studien über die endotrophen Mycorrhizen. Jahrb. Wiss. Bot., **37:** 643-684.

*Shibata K. and Tahara M. (1917). Studien über die Wurzelknöllchen. Jahrb. Wiss. Bot., **31:** 157-182.

Simonet P., Hauret J., Normand P., Bardin R. and Moiroud A. (1986). Localization of *nif* genes on a large plasmid in *Frankia* sp. strain ULQ132105009. Mol. Gen. Genet., **204:** 492-495.

Simonet P., Normand P. and Bardin R. (1988). Heterologous hybridization of *Frankia* DNA to . *Meliloti* and *K*. *pneumoniae nif* genes. FEMS. Microbiol. letters, **55**: 141-146.

Simonet P., Normand P., Hirsch A.M. and Akkermans A.D.L. (1990a). The genetics of *Frankia* actinorhizal symbiosis. *In* The Molecular Biology of Symbiotic Nitrogen Fixation, P.M. Gresshof (ed). CRC Press Inc. Florida, USA. pp. 70-109.

Simonet P., Normand P., Moiroud A. and Bardin R. (1990). Identification of *Frankia* strains in nodules by hybridization of Polymerase Chain Reaction products with strain - specific oligonucleotide probes. Arch. Microbiol., **240:** 153-235.

Simonet P., Grosjean M.C., Misra A.K., Nazaret S., Cournoyer B. and Normand P. (1991). *Frankia* genus - specific characterization by polymerase chain reaction. Appl. Environ. Microbiol., **57:** 3278-3286.

Simonet P., Bosco M., Chapelon C., Moiroud A and Normand P. (1994). Molecular characterization of *Frankia* micosymbionts from spore-positive and spore-negative nodules in natural alder stand. Appl. Environ. Microbiol., **60:** 1335-1341.

*Stackebrandt E. (1985). Phylogeny and phylogenetic classification of prokaryotes, *In* Evalution of Prokaryotes, K.H.Schleifer and E. Stackebrandt (ed). Academic Press. London. pp. 309-334.

Tjepkema J.D. and Schwintzer C.R. (19900. The Biology of *Frankia* and Actinorhizal Plants. Academic Press Inc. California.

Torrey J.G. (1987). Endophyte sporulation in root nodules of actinorhizal plants. Physiol. Plant., **70:** 279-288.

Tzen S.S. and Torrey J.G. (1989). Spore germination and the life cycle of *Frankia in vitro.* Can J. Microbiol., **35:** 801-806.

Wheeler C.T. and Miller I.M. (1990). Current and potential uses of actinorhizal plants in Europe. *In* The Biology of *Frankia* and Actinorhizal Plants, C. R. Schwintzer and J.D. Tjepkema (eds). Academic press inc. California, USA. pp. 317-338.

*Woronin M. (1866). Uber die bei der Schwarzerle (*Alnus glutinosas*) und bei der gewohnlichen Gartenupine (*Lupinus mutabilis*) auftretenden Wurzelanschwellungen. Mem. Acad. Imp. Sci. St. Peterburg. Ser 7. T.,**10(6):** 1-13.