CHAPTER 1

Introduction

Symbiotic association, a perfect example of division of labour between two organisms, the outcome of which may lead to endeavors like nitrogen fixation. Reduction of atmospheric N_2 to ammonia and its further assimilation into amino acids and other bio-molecules enables gaseous nitrogen to incorporate into life processes. As all organisms need Nitrogen to survive, nitrogen fixation is probably the second most important biochemical pathway after CO_2 fixation. However, the ability to fix nitrogen is found only in one biologic kingdom, the Prokaryota (Sprent & Sprent 1990). Thus, other organisms have exploited the ability of prokaryotes to fix nitrogen by establishing various types of interactions (Werner 1992). Cyanobacteria and plant-microbe symbioses are considered to be among the major milestones in evolution of life on Earth, bringing together the two most essential biochemical pathway, carbon fixation and nitrogen fixation. There occur two main types of symbioses between nitrogen-fixing bacteria and vascular plants: one between *Rhizobium* and leguminous plants, and the other between *Frankia* and actinorhizal plants (Wall 2000). A large number of woody dicotyledonous plants making symbiotic association with actinomycetes, belonging to the genus *Frankia* are called actinorhizal plants (*viz. Alnus nepalensis, Eleagnus pyriformis, Myrica nagi, Casuarina eqisetifolia, Coriaria nepalensis, and Hippophae sp.*etc).

The rhizobia-legume symbiosis involves more than 1700 plant species of the family Fabaceae (Leguminosae) distributed in three sub-families: Mimosoideae, Ceaesalpinoideae, and Papilionoideae (Wall 2000) with bacterial partners belonging to the family Rhizobiaceae *(Rhizobium, Azorhizobium, Sinorhizobium, Bradyrhizobium,* and *Mesorhizobium*) (Crespi & Galvez 2000). Unlike *Rhizobium*, *Frankia* form symbiosis with 25 different genera of Dicotyledonous mostly woody plants belonging to 8 families. These plants called actinorhizal plants comprise more than 220 species symbiotically associated with the filamentous actinomycetes *Frankia.* However, in all cases of *rhizobia*-legume or *Frankia*-actinorhizal symbiosis, a new plant organ, the nodule, is developed in which the bacteria proliferate, express the most vital enzyme, the nitrogenase and fix nitrogen in to ammonia. These compounds are then assimilated and transported to the rest of the plants (Hirsch 1992;

Pawlowski & Bisseling 1996; Franche et al. 1998). Various genes of both host and bacteria are involved in the process of nodulation and nitrogen fixation. In some cases shoot nodules are found but they may be adventitious roots associated with micro-symbiont to form nodule, very similar in construction with root nodules but are located above the soil. Distinctive features of actinorhiza in anatomy with legume nodules are: 1) legume nodules have an innermost infected tissue bounded by nodule parenchyma and peripheral vascular bundles, where as actinorhizal nodules are characterized by a central vascular bundle and peripheral infected tissue surrounded by cortical nodule parenchyma; 2) actinorhizal nodules are ontogenically related to roots whereas legume nodules bear shoot-like anatomy.

Most of the Actinorhizal plants are woody shrubs or trees and are perennial dicots, except for *Datisca*, which has herbaceous shoots. They have in common a tendency to grow in marginally fertile soils and they often serve as pioneer species early in successional plant community development (Schwintzer & Tjepkema, 1990). Actinorhizal plants are some of the first plant species to colonize the damaged environment caused by natural disaster such as landslides and volcanic eruptions or erosions (Burleigh & Dawson, 1991). Pollen distributions, determined from analysis of marine sediment cores, show that actinorhizal plants colonize de-glaciated soils during times of major climatic change (Heusser & shackleton, 1979).Once established on a site, actinorhizal plants can fix N_2 and add nitrogen to the soil in the form of leaf litter and dead root tissue. Actinorhizal plants, hence, build up soil organic matter and create a more favorable habitat for other plants and soil organisms (Cracker and Major 1955; Lawrence et al. 1967; Olff et al. 1993; Chapin et al. 1994; Aplet 1990). Since, these plants often flourish on marginal soil; they have current and impending application in reclaiming and conditioning soils, producing timber and pulp and performing as windbreak, ornamental and fuel wood plants, etc. Globally they have potential for integrating in to schemes for addressing issues of reforestation. The relationship between *Frankia* and host species contributes significantly to global nitrogen cycles. Actinorhizal plants provide nitrogen rich organic matter and are often fundamentals to the dynamics and biodiversity of terrestrial ecosystems. Actinorhizal

plants have potential relevance in increasing the soil fertility of nitrogen deficient sites and thus set foundations for a more abundant ecosystem (Benson & Silvester, 1993; Lawrence et al. 1967; Conrad et al.1985; Hibbs & Cromack 1990; Thilenius 1990). Examples of important early successional actinorhizal plants in the western U.S. include sweetgale (*Myrica gale)* and sitka alder (*Alnus viridis*) in coastal wetlands of Alaska (Thilenius 1990), numerous species of *Ceanothus* in chaparral, forest and mountain shrublands (Hickey end Leege 1970; Leege 1979; Conrad et al. 1985), the genus *Alnus* in the Pacific Northwest (Hibbs & Cromack 1990), and species of *Dryas* in arctic and alpine habitats in Alaska (Cracker & Major 1955, Lawrence et al. 1967). Alders and dryads were common colonizers of glacial till following the retreat of continental glaciations in the northern hemisphere (Ritchie 1987).

Actinorhizal plants have worldwide distribution. Most of them are temperate plants. However, some members of Casuarinaceae and Myricaceae are native to the tropical regions. Species of *Alnus* or *Elaegnus* are found in hilly areas of the tropics.

Actinorhizal plants, *Alnus nepalensis* in particular, prefers moist, cool climates with mean annual temperature of 13-26°C and mature trees are tolerant to frost. This plant can grow at high altitudes (up to 3000 m) in both temperate and subtropical regions, with annual rainfall 500 to 2500 mm and a dry season up to about 6 months long. It is the most droughts tolerant of the *Alnus* species but best growth is obtained in areas where the mean annual rainfall exceeds 800 mm and the relative humidity is higher than 70%. They prefer soils that are moist and well drained, but not waterlogged. They do not require high soil fertility but prefers permeable soils and does poorly on dry, exposed ridge tops.

Alnus nepalensis is native to Pakistan, eastern Nepal, Bhutan, northern India, south-western China, upper Myanmar and parts of Indochina. This plant has been introduced to various countries in Africa, Central America and South-East Asia. It has been included in species trials in Burundi (Brunck et al., 1990), in Uganda (Okorio et al., 1994), in the highlands of Java (1350 m altitude) (Rostiwati & Suriamihardja, 1987), and in agroforestry trials in Bolivia (Mahboubi et al., 1997; Baker & Schwintzer, 1990).

Alders are pioneer species favored by high light levels and exposed mineral soils; in addition, their ability to fix atmospheric nitrogen facilitates establishment on geologically young or disturbed sites with low levels of soil nitrogen (Harrington et al., 1994).

The identity of the actinorhizal root nodules endophytes as actinomycetes was established in 1964 when the electron microscopy revealed the prokaryotic structure of the microorganism in *Alnus glutinosa* and *Myrica serifera* root nodules (Becking *et al.* 1964). A more detailed description became available only in 1978, when Torrey group first successfully isolated *Frankia* strain (CpI1, now known as HFPCI1 from *Comptonia peregrina* nodules) in pure culture (Callaham et al. 1978). The field of research involving *Frankia* and actinorhizal plants has undergone rapid expansion with this event. Since, pure *Frankia* strains cultivatable *in vitro* are available; it is now feasible to apply the modern techniques of microbiology, physiology, biochemistry, molecular biology and genetics to this group of nitrogen fixing actinomycetes. .

The microsymbiont of actinorhizal plants was first referred to as *Frankia* in 1888 by Brunchorst and was later classified as an actinomycete after studies by Krebber in 1932 (Quispel 1990). *Frankia* belongs to the family Frankiaceae in the order Actinomycetales. The genus comprised of gram-positive to gram-variable strains (Lechevalier & Lechevalier 1990). *Frankia* differentiate into three cell types: vegetative hyphae, sporangia, and vesicles. These different cell types can be produced in pure culture, in planta, and presumably in soil. *Frankia* grows as a filamentous colony on agar plates and being micro-aerophilic to reluctantly aerophilic are cultured in liquid media (Lechevalier & Lechevalier 1990). In batch static cultures, the bacteria grow as threadlike submerged colonies without aerial or floating growth, and when grown under nitrogen limitation, form three characteristic cell types: filaments, vesicles, and multilocular sporangia (Akkermans & Hirsch 1997; Benson & Silvester 1993). Vegetative cells are generally poorly branched. Vesicles are the site of nitrogenase expression and nitrogen fixation (Huss-Danell and Bergman 1990; Tisa and Ensign 1987). They exclude oxygen, thereby protecting nitrogenase (Parsons et al., 1987) and exhibit a distinctive metabolism (Tisa 1998; Tisa and Ensign 1987; Tisa

and Ensign 1988). Vesicles are usually spherical in cultivated *Frankia,* whereas in nodules they often assume different shapes (spherical, elliptical, club-shaped etc.). Vesicles can also be septate or nonseptate. The third type of differentiated structure, the multilocular sporangia, is filled with spores, which can remain for long periods in dry soil as infective particles (Tortosa & Cusato 1991). On the basis of the presence or absence of sporangia within a root nodule, *Frankia* strains have been classified as either spore$^+$ or spore$^-$ (Schwintzer 1990). Spore$^+$ strains appear to be much more infective than spore$^-$ strains (Normand & Lalonde 1982); both have been characterized at the molecular level (Simonet et al. 1994). All three cell types can be found in the symbiotic state (Newcomb & Wood 1987) with few exceptions. Cultivated *Frankia* cells behave as heterotrophic aerobic bacteria with doubling times of 15 h, compared with 3 h for rhizobia. Nevertheless, the growth of *Frankia in planta* seems to be unrestricted, because timing of root infection, nodule development, and host cell infection are similar to those of rhizobia-legume nodules. Thus, the difficulties of growing *Frankia* in culture or isolating *Frankia* from some plant species reflect our limited knowledge of isolation and growth requirements.

Like other N_2 fixing microorganisms, members of actinorhizal genus *Frankia* can reduce atmospheric nitrogen with the help of nitrogenase enzyme. The site of nitrogenase is in the vesicle. In vesicles, protection against oxygen toxicity at a high partial oxygen pressure is provided by the build up of lipid containing layers at the surface (Parsons *et al.* 1987). Freeze fracturing technique has been used to demonstrate this unique feature (Harriot *et al.*1991; Parsons *et al.*1987). Actinorhizal nodules show an optimum nitrogen activity at around 20 K Pa of oxygen and significant inhibition above 25 K Pa of oxygen (Rosendahl; *et al.*1988; Silvester *et al.* 1988). *Frankia*e growing in artificial media produce the same general morphological structure that they produce *in planta, including* sparsely branched hyphae, vesicles and multilocular sporangia containing non-motile spores. Sporangiospores are unique among actinomycetes. They are surrounded by multiple membranous layers; those are visible by electron microscopy. Though, in actinorhizal nodules, hyphae and vesicles are the most prominent features, spores and sporangia of the endophyte have also

been detected in different species (Schwintzer 1990; van Dijk 1978; Van Dijk and Merkus, 1976). The anatomy of nodule tissue shows vesicles those, may be globose, pear shaped or elongated (Torrey, 1985). The vegetative phase of *Frankia* displays septate, branched, filamentous hyphae, producing a mycelial mat when grown on solid media (Benson and Silvester, 1993). The vegetative form is exhibited in pure culture, infection and proliferation within the plant and presumably saprophytic life within the soil.

Although *Frankiae* are exacting in their growth requirements, they do not need complex media to grow well. A simple salt solution supplemented with organic acid such as Pyruvate or Propionate as a carbon source often is sufficient since *Frankiae* can fix nitrogen *in vitro* (Lechevalier and Lechevalier, 1990).

Actinorhizal plants are strong competitors of legumes in respect to the amount of nitrogen they fix on global basis. Furthermore, the ability to dispense large quantities of infective and effective strains for various host plants of economic importance for forestry is also increasing the interest both in evaluating and optimizing various combinations of host strains and in understanding the involvement of both partners in order to manipulate the symbiotic system in future (Baker 1987).

However, the study of *Frankia* in pure culture is always a difficult criterion. The difficulties in studying *Frankia* in pure culture as highlighted by Benson and Schultz (1990) are primarily related to its pleiomorphic growth form and are magnified in ecological studies of *Frankia*. *Frankia* cannot be directly isolated from soil and counted on plates, because they are very slow growing and poor competitors for readily available carbon sources. There are no selective media available for *Frankia* till date. Even isolation from nodules is problematic because of the inability of *Frankia* strains to compete with other contaminating microorganism. Besides, we cannot discard the idea that some *Frankia* strains may be non-culturable.

Previously the heterogeneity among *Frankia* strains have been studied on the basis of total cellular protein and isozyme patterns (Benson and Hanna, 1983; Benson *et al.*, 1984; Gardes and Lalonde 1987; Gardes *et al.*, 1987). Benson and Hanna

(1983) performed a study on *Alnus incana* stand and they placed 43 nodule isolates into five groups based on one-dimensional SDS- polyacrylamide gel electrophoresis of total cell proteins.

Previous attempts were limited due to difficulties with growing enough cellular material for DNA extraction and also by the low efficiency of classical lysis techniques of *Frankia* cells. Later by exploiting certain enzymes like lysozyme in combination with a drastic extraction method the Lyon group tried a number of DNA isolation procedures. However, the use of achromopeptidase by Simonet et al. (1984) gave better results.

Previous work on *Frankia* (Reddell and Bowen, 1985; Sougoufara et al., 1992) revealed that host may play an important role than the microsymbiont during the commencement and subsequent development of symbiosis. Past experiments, pertaining to physiological data, point towards a direct involvement of the host, but suffer from lack of molecular evidence. Therefore further studies are required in this field.

Fortunately, now we have the molecular tools to detect and analyze *Frankia* in soil and in nodules without the need for culturing the bacteria. Polymerase chain reaction (PCR) techniques using primers specific to 16S rRNA genes, intergenic region of 16S-23S rRNA, intergenic region of *nifD-nifK* genes, or rep-PCR primers etc. have been applied to *Frankia* isolated from almost all actinorhizal plant genera (Benson et al. 1996; Clawson et al. 1998; Jamann et al. 1993; Jeong et al. 1999; Murry et al. 1997; Nalin et al. 1995; Nazaret et al. 1991; Normand et al. 1996). Sequence analysis of the PCR amplified 16S r-RNA gene has greatly facilitated the systematic, evolutionary and ecological studies of various microorganisms (Olsen *et al.* 1986; Woese *et al.*1987). This technique is particularly valuable for non-culturable microorganisms and has been extensively used for the elucidation of the phylogenetic relationships of various microorganisms (Giovannoni *et al.* 1990), including *Frankia* populations within the alder root nodules (Mirza *et al.* 1992; Nazaret *et al.* 1991; Nick *et al.* 1992). Defined molecular phylogeny groupings of *Frankia* are obvious from these studies (Clawson and Benson 1999; Mirza et al. 1994; Rouviere *et al.* 1996).

Objectives

The main objective of the present research work was to characterize and hence investigate phylogenetic relationships and best possible Alder-*Frankia* symbiosis of uncultured *Frankia* strains from the root nodules of *Alnus nepalensis*, native of Eastern Himalayas by using modern molecular biology techniques. Research on actinorhizal plant, *Alnus nepalensis*, was initiated due to its importance in reclaiming and nourishing soil, acting as source of timber and fuel-wood plant, preventing soil erosion and addressing the issues of polydenitrification globally.

Considering the present standing of *Frankia* and actinorhizal plants and critical gap areas in this field chiefly with respect to Sikkim and Sub- Himalayan West Bengal, the aims of the present study are:

- Collection of alder-*Frankia* germplasm from different parts of aforementioned regions.
- Isolation, purification and genetic screening of axenic cultures as well as nodular acquaintances of diverse *Frankia* strains from Sub-Himalayan West-Bengal and Sikkim.
- Substantiation of the integrity of the culture by PCR amplification of *Frankia* specific DNA sequences and physiological tests.
- Cloning of altered PCR amplicons for further studies.
- Learning the influence of host on nitrogen fixation rate of in-nodule *Frankia*.