MOLECULAR TYPING OF *FRANKIA* IN THE FIELD COLLECTED ACTINORHIZAL ROOT NODULES FROM DIFFERENT ALTITUDES IN SIKKIM

By

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IN FULFILMENT OF THE DEGREE OF
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Dedicated to my parents

The North-Eastern Hill University

August, 2006

DECLARATION

I, Anindita Khan, hereby declare that the subject matter of this thesis entitled "Molecular typing of *Frankia* in the field collected Actinorhizal root nodules from different altitudes in Sikkim" 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 anybody 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.

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CHAPTER 1

INTRODUCTION

The growth of all organisms depends on the availability of mineral nutrients, and none is more important than nitrogen which is required in large amounts as an essential component of proteins, nucleic acids and other cellular constituents. Though there is an abundant supply of nitrogen in the atmosphere, molecular nitrogen is metabolically unavailable directly to higher plants and animals. The presence of a triple bond between the two nitrogen atoms of a molecule makes it almost inert. Nitrogen must be converted into ammonium (NH_4^+) or nitrate (NO_3^-) ions before it could be used by plants and animals. Conversion of molecular nitrogen to ammonium or nitrate forms is also called as nitrogen fixation. Only few microorganisms can 'fix' atmospheric nitrogen, making all other living organisms dependent on them for their requirements of 'fixed' nitrogen.

Micro-organisms that fix nitrogen are called diazotrophs. Biological nitrogen fixation is brought about by two types of micro-organisms – non-symbiotic or free living and symbiotic. The free living non-photosynthetic diazotrophs require a chemical energy source for bringing about nitrogen fixation, whereas the free living photosynthetic diazotrophs utilize light energy for the purpose. Free living micro-organisms like *Scytonema*, *Rivularia* or *Klebsiella* fix nitrogen utilizing fixed carbon as energy source. They therefore contribute very little fixed nitrogen to agricultural crops.

On the other hand diazotrophs that live in close proximity to plant roots and obtain fixed carbon from the plants are symbiotic or associative nitrogen-fixing microorganisms. Microorganisms like *Rhizobium* and *Frankia* form symbiotic association with host plant(s) and utilize fixed carbon supply of the host for fixing atmospheric di-nitrogen. These microorganisms make a substantial contribution of fixed nitrogen to agriculture and forestry. It has been estimated that *Frankia* contributes about 2-362 kg N/ha/yr while the estimated contribution of Rhizobium-Legume symbiosis is about 24-584 kg N/ha/yr. (Shantharam and Mattoo, 1997).

Since the *Frankia* symbiosis results from an actinomycetic invasion of plant roots, it has been termed as "actinorhizal symbiosis" (Benson, 1988). Accordingly, the plants nodulated by *Frankia* are called "actinorhizal plants". Although these plants are taxonomically diverse, they have some common features. For example, all of them are dicotyledonous and perennial angiosperms (Baker and Schwintzer, 1990). Actinorhizal plants belong to four subclasses, eight families, twenty-five genera and more than 220 species (Wall, 2000). Well known genera are *Alnus* (Betulaceae), *Myrica* (Myricaceae), *Casuarina* (Casuarinaceae), *Elaeagnus, Hippophae* (Elaeagnaceae), etc. *Frankia* have attracted attention recently because they form root nodules on a broad range of non-leguminous plants and because such nodules fix N_2 as effectively as rhizobial nodules.

Among actinorhizal plants, the two genera *Alnus* and *Casuarina* exhibit highest nitrogen fixing potential (Dommergues, 1996) and *Alnus* is the most extensively studied among them. *Alnus* species are important among actinorhizal plants as pioneers in ecological succession of skeletal soils (Lawrence, 1951; Crocker and Major, 1955; Damière *et al.,* 1986). They are widely used for intense forest management (Tarrant and Trappe, 1971; Borman and Gordon, 1984), biomass production (Zavitkovski *et al.*, 1979) and regeneration of disturbed lands (Heilman, 1982). It is thought to have originated in Indo-China region (Furlow, 1979). About 47 species of *Alnus* are known (Swensen and Mullin, 1997). In India, only two species are found (*A. nepalensis* and *A. nitida*) and they are naturally distributed throughout the temperate Himalaya. *A. nepalensis* is confined to the higher elevations of Meghalaya and Arunachal Pradesh, and *A. nitida* is found in

Himachal Pradesh. Trees of *A. nepalensis* are also found in some locations of Nagaland and Tamil Nadu (Varghese, 2000).

The genus *Hippophae*, commonly known as sea buckthorn is another actinorhizal plant which forms symbiotic association with *Frankia.* A fascinating plant species, it is a very attractive ornamental shrub. It has silvery deciduous leaves and colourful orange berries that persist through most of the winter. It is a native of Eurasia and used by humans for centuries (Akulinin, 1958; Ge *et al.*, 1985). Among all the species of this genus, *Hippophae rhamnoides* is the most widespread. It has been divided into approximately eight geographically separated subspecies, but some scientists think that some of these deserve the rank of species (Small *et al.*, 2000). The unusually hairy *H. gyantsensis* occurs only in a restricted part of Tibet adjacent to Sikkim (Small *et al.*, 2000).

Hippophae is believed to be a colonizer of open habitats. In India their distribution is strictly restricted to the higher altitudes of the Himalayas. The highly efficient relationship with *Frankia* results in the improved root growth which enhances the entire soil ecosystem.

Sea buckthorns have great economic value. The berries, leaves and seeds have tremendous nutritional and medicinal value. It has three to sixteen times as much vitamin C as Kiwi fruit. Its superoxide dismutase content of the fruit juice is four times higher than that found in Ginseng. It contains twenty four minerals, eighteen amino acids and eight vitamins (Small *et al.*, 2000). Sea buckthorn is also useful as soil enhancer, pollution reducer, source of firewood, and as a landscape management tool (Li and Schroeder, 1996).

The actinorhizal plants are found primarily in the temperate zone. Only some species of the Casurinaceae and the Myricaceae can be considered truly tropical. Some species of

Alnus and *Elaeagnus* are also found in the tropical zone but these species are restricted to the higher elevations of mountain regions, where the climate is essentially temperate. In high latitude countries such as Scandinavia, Canada and New Zealand conditions are not favourable for legumes to thrive. However, actinorhizal plants are abundant and capable of vigorous growth (Silvester, 1977).

These plants involved in symbiosis with *Frankia* are important pioneer species in nitrogen poor soils or disturbed environments (Benson *et al.*, 1984). They are extremely useful for rehabilitation of degraded and salt affected soils, where other plants may fail to grow. The high nitrogen content of the leaf litter of actinorhizal trees increases soil fertility and can pave the way for diversification of species within the ecosystem. Recent genetic studies show that possibly the eight different tree families of actinorhizal host plants developed the capacity for symbiosis at separate stages during evolution (Swensen, 1996; Myrold *et al.*, 1999).

Though the actinorhizal association is analogous to the much better studied *Rhizobium*-legume symbiosis, very little is known about *Frankia*, especially its ecology and genetics. Thus there is a need for obtaining basic information about the population sizes and diversity of *Frankia*. It may be noted that the first successful isolation of *Frankia* was reported only in 1978, when strain CpI1 was obtained from *Comptonia peregrina* by Callaham *et al.* (1978).

Over the past few decades, agriculture in the developed world has become increasingly dependant on industrially fixed chemical fertilizers and pesticides for achievement and maintenance of the high yields possible with modern crop types. In recent years, the adverse effects of these chemicals on the rural population, the environment and on food safety, has urged the need for changes in agricultural production methods, with the objectives of sustainability, economic production, conservation of natural resources, and reduction in the use of synthetic chemicals. The challenge is to institute sustainable methods without compromising food-production levels, particularly in view of the need to increase agricultural yields worldwide to accommodate population growth.

There are several significant environmental reasons to seek alternative to chemically fixed nitrogen fertilizer. Chemical fixation of atmospheric nitrogen affects the balance of the global N_2 cycle, pollutes ground water, increases the risk of chemical spills and increases atmospheric nitrous oxide- a potent “green house” gas. On the other hand, biological nitrogen fixation offers an economically attractive and ecologically sound means of reducing external nitrogen input and improving the quality and quantity of internal resources.

Forest trees are essential for human society. They provide fuel, fiber, building materials, food and medicines among other things. Forests stabilize environments and trees are cornerstones of ecosystems. The importance of nitrogen-fixing actinorhizal plants in forestry has been recognized (Montpetit and Lalonde, 1988). These plants are widely used in such practices as reforestation, land reclamation and biomass production (Fessenden 1979; Gordon *et al.*, 1979; Ponder, 1983; Tarrant *et al.*, 1971).

The genetic resources of forest trees must be conserved to maintain and improve our forests. Various classification systems have been used to categorize modern biotechnologies used in forestry sector, mainly because of the potential genetic gains they could confer. This can be categorized as – biotechnology employing molecular markers, biotechnology aimed at enhancing plant propagation and biotechnology for modifying the genome of forest tree species. Application of biotechnologies in forests has been seen as a unique opportunity for obtaining new information on the extent, patterns and functioning of tree genetic diversity and for providing new tree varieties and reproductive materials adapted to changing environmental, social and economic environments (Fenning and Gershenzon, 2002).

Biotechnology can provide tools for the identification of superior genotypes through the characterization of DNA markers. The same tools are used to study the genetic diversity of tree populations which is a component of biodiversity and is important in ensuring the sustainability of the forest resource.

Frankia-actinorhizal symbiosis has attracted a great attention in Biological Nitrogen Fixation research recently. Prior to this our understanding of the molecular genetics of *Frankia* was restricted because of the fact that frankiae have slow growth rates, and there was lack of availability of a suitable vector for introduction of alien DNA into *Frankia* cells. *Frankia* strains have tremendous diversity at molecular level as shown by DNA-DNA hybridization of total cellular DNAs. Moreover, plasmids have been found in some frankiae. Several of the *nif* genes have been identified in *Frankia* but progress in identifying *nod* genes has been limited.

Actinorhizal symbiosis is such an association where both the host and the microbe have very important roles to play. Therefore, a superior and efficient host microbe relationship could positively affect nitrogen-fixing capacity of the *Frankia* strain (Chauhan, 2000). And for establishing this superior and efficient relationship, most infective, effective and competitive *Frankia* strains have to be selected, which in turn require investigations on diversity existing within the species.

Study of genetic diversity of *Frankia* would become useful if this information is used in enhancing its natural abilities. There exists tremendous potential if we are able to enhance the nitrogen fixing ability of *Frankia* and are able to extend its host range to include some horticultural trees. However, we must understand the effect of ecological parameters on molecular constitution and behaviour before we set out to achieve the above. It is in this context that the present study was taken up.

Objectives

The present study was taken up to study the impact of certain ecological parameters on distribution of different strains at different sites. To achieve this broad objective, the following specific tasks were laid out:

1. Molecular typing of *Frankia* strains present in the field collected nodules of some genera of actinorhizal trees from Sikkim.

2. To study relationship, if any, between ecological parameters (different soil characteristics, altitude etc.) and molecular diversity of *Frankia*.