CHAPTER 2

Review of Literature

2.1. History of *Frankia* research

Nitrogen is the mineral nutrient, essential to all living organisms, which most often limits plant production. Though gaseous atmosphere is 80% dinitrogen (N2), it cannot be utilized as such. This is because the triple bonded structure N≡N is extremely stable and chemically very non-reactive on account of the high-energy bond of the molecule. Nitrogen from the atmosphere can be fixed abiologically (industrial synthesis, ultraviolet irradiation, lightning) as well as biologically. Industrial synthesis contributes about 60 million tones of nitrogen per year for fertilizer uses whereas biological nitrogen fixation contributes about 100-175 million tones per year. Biological nitrogen fixation (BNF) accounts for 65% of nitrogen currently utilized in agriculture, and will be increasingly important in future crop productivity, especially for sustainable systems (Sen & Bajwa. 2002).

Biologically the ability to fix molecular nitrogen, occur, in a diverse groups of microorganisms that exist, either as free-living diazotrophs or in symbiotic associations with plants (Young 1992). As free-living diazotrophs only prokaryotes (bacteria, blue green algae and actinomycetes) and archaebacteria can convert dinitrogen to ammonia. Symbiotic nitrogen fixing bacteria include the cyanobacteria, the genera *Rhizobium,Bradyrhizobium, Azorhizobium, Sinorhizobium, Mesorhizobium and Frankia* (Paul and Clark 1996; Brock 2000). The root-nodule is the most important symbiotic association between nitrogen fixing microorganisms and higher plants (Akkermans and Houwers 1979; Dixon and Wheeler 1986).

Unfortunately, the study of root nodules of non-leguminous plants was shrouded in the mist of misconceptions for a long period. Between 1886 and 1888, Hellrigel and Wilfarth published two papers, which brought an end to a 10-year-old controversy about sources of nitrogen for growth of plants and opened up new frontiers of plant microbial sciences. They showed that legume nodules fixed atmospheric nitrogen. They also gave an idea about nodule inducing ferment, showed that it was more or less specific, was killed by moderate heat, and harmed by drought. Nitrogen fixed in a nodulated legume was not immediately available to the neighboring plants and that small quantities of combined nitrogen did not affect nodulation whereas larger amount was inhibitory

(Quispel 1988). Hellrigel and Wilfarth gave the idea of the difference between nitrogen users and nitrogen accumulators. Alders were initially pushed into the nitrogen-accumulating group. However, Hiltner proved that actinorhizal plants could utilize atmospheric nitrogen. In all cases of rhizobia-legume or *Frankia*-actinorhizal symbioses, a new plant organ is developed in which the bacteria differentiate, express the enzyme nitrogenase, and fix nitrogen into ammonia. These compounds are then assimilated and transported to the rest of the plant. Legumes and actinorhizal plants develop root nodules as a consequence of compatible plant-bacteria interactions through the switching on and off of genes in both genomes to establish a newly developed, shared structure. In some cases, so-called shoot nodules are formed (Prin *et al.* 1992), but these aerial nodules are modified adventitious roots, having the same structure as root nodules but are located above the soil or the stems.

Although both *Frankia*-actinorhizal plant and *Rhizobium*-legume symbioses have been known for many years to benefit soil fertility, the nature of these nitrogen-fixing symbioses and the microsymbionts involved were discovered at the end of 19th century (Quispel 1990). Various anatomical studies, cytological studies and Studies by Hellrigel and Wilfarth have been done to compare leguminous and actinorhizal microsymbionts. Actinorhizal and legume nodules can be easily distinguished at the anatomical level (Pawlowski and Bisseling 1996). Legume nodules have a central infected tissue surrounded by nodule parenchyma and peripheral vascular bundles, whereas actinorhizal nodules are characterized by a central vascular bundle and peripheral infected tissue surrounded by cortical nodule parenchyma. Legume nodules have been proposed to have a shoot like anatomy, whereas actinorhizal nodules are ontogenically related to roots. In another study the legume root nodule bacteria isolated by Beyerinck failed to infect the non-leguminous plants which led to the conclusion that the two microsymbionts are two different microorganisms.

Study of the actinomycetes induced nodule symbiosis has lagged behind research on legumes largely because of the repeated failure of attempts to isolate and grow the bacterial endosymbiont in pure culture by many workers. Although until 1978 isolation and cultivation of *Frankia* strains were generally considered to be impossible, since that

year Callaham *et al.* (1978) reported the first successful isolation of *Frankia* from the root nodules of *Comptonia peregrina* (L.). Later on Sarma *et al.* (1998) have described a more convenient method for isolation of *Frankia* by Calcium-alginate beads both for isolation of *Frankia* from nodules and generation of single spore cultures. Borthakur *et al.* (1996)&(1997) observed germination of spores into active colonies even when incubated in nitrogen free medium. To date, *Frankia* strains from a number of other actinorhizal species are available in pure culture (Lechevalier 1986; Benson and Silvester 1993) and their growth requirements have been optimized (Akkermans *et al.* 1983; Blom 1982; Meesters *et al.* 1985; Tjepkema *et al.* 1980).

2.2. *Frankia*- The microsymbiont

Frankia belongs to the family Frankiaceae of the order Actinomycetales. The genus name *Frankia* was proposed in 1886 by J.Brunchorst to honor his mentor, A.B Frank a Swiss microbiologist. Later, Krebber classified it as actinomycetes in 1932 (Quispel 1990). The genus *Frankia* is comprised of gram-positive and gram-variable actinomycetes (Lechevalier and Lechevalier 1990). The first cultured *Frankia*, isolated from *Alnus* root nodules was reported by Pommer (1959), but unfortunately the culture was lost. In 1978, the first successful isolation of *Frankia* was reported from *Comptonia peregrina* root nodules (Callaham *et al.* 1978), beginning a new era in actinorhizal symbiosis research (Quispel 1990).

2.2.1. Taxonomy of the genus *Frankia*

Following the successful propagation of the first isolate, more and more strains became available, and problems of nomenclature and taxonomy became a matter of concern.

Since Brunchorst and Frank considered the microorganism *Frankia* to be a fungus, the name was not generally used until Becking, in 1970, revived it and redefined the genus as consisting of members of the Actinomycetales in a new family, Frankiaceae (Becking 1970). Becking further proposed that 10 species be established (*Frankia alni*,

F.elaeagni, F.brunchorstii, F.discariae, F.casuarinae, F.ceanothi, F.coriariae, F.dryadis, F.purshiae, and F.cercocarpi) based on the source plant, and, since numerous attempts to obtain the organisms had failed, he described the members as "obligate symbiotic organisms". *Frankia* strains still constitute a separate family within the order Actinomycetales. In Bergey's Manual of Systematic Bacteriology, vol.4 (1989), they are included among "actinomycetes with multilocular sporangia" (Lechevalier & Lechevalier 1989). Other genera in this group include *Geodermatophilus* and *Dermatophilus*. Recent 16S rRNA sequence analyses of representatives of these genera have revealed a relatively close phylogenetic relationship between *Frankia* and *Geodermatophilus* but not *Dermatophilus*; an invalidly described isolate from the Black Sea called "*Blastococcus*" also clustered with *Frankia* (Hahn *et al.* 1989). Thus, future descriptions of the family Frankiaceae should include members of these genera. The family Dermatophilaceae will consequently contain only the genus *Dermatophilus* (Hahn *et al.* 1989). The taxonomy of *Frankia* is being reevaluated because of the uncertainty regarding species recognition and the confusion arising from trivial strain designations (Lechevalier 1983), the initial classification proposed by Becking (1970) has been replaced with a system of registry numbers. The availability of numerous strains (Baker, 1982) helped in the clarification of the relationships among various members in this unique family Frankiaceae. It should be noted that only one genus and no species were recognized until 1988. This absence of recognition of species within the genus *Frankia* was mainly due to vast variations in the various morphological and physiological characters normally used for taxonomic purposes.

Since 1978 (Callaham *et al.* 1978) an increasing number of *Frankia* strains have been isolated, and attempts have been made to classify the strains. So far all the strains have been classified within the genus *Frankia*. This taxon is characterized by:

a. The ability to nodulate plants;

b. The ability to fix nitrogen;

c. Its unique morphological properties, vesicles and sporangia;

d. The presence of sugar, 2-o-methyl-D-mannose (Mort *et al.* 1983);

e. The presence of cell wall type III and phospholipid content of type I of actinomycetes (Lechevalier and Lechevalier 1990);

f. A high G+C% in the range of 68- 72%

The characters employed in actinomycete taxonomy in the past have been the size and shape of sporangia or spores, and production of cellular or soluble pigment. For *Frankia* these characters are not considered sufficiently stable to be useful (Lechevalier 1984). According to Weber *et al.* (1988) the morphological characters are stable but too diverse for identification use. A combination of different observations (like Sporangial size, frequency and shape; pigment production; urease activity and acetate decarboxylation) can be used to separate some of the strains, but do not allow general identification of individual *Frankia* strains. It should be noted that, although the available *Frankia* isolates can belong to various host specificity groups, this character is not used in the actual because some *Frankia* strains can infect two host specificity groups, such as *Alnus* and *Elaeagnus* groups and because some non infective strains of *Frankia* are known to exist. As mentioned above one stable character is the presence of a unique sugar, identified as 2-O- methyl D-mannose, which is a unique trait of Frankiaceae family (Mort *et al.* 1983).

Related strains can also be distinguished on the basis of electrophoretic separation of isozyme. Gardes *et al.* (1987) characterized *Frankia* strains by electrophoretic separation of isozyme of eight different enzymes. They analyzed diaphorase, leucine aminopeptidase's, phosphoglucose isomerase, esterase, maltate dehydrogenase, phosphoglucomatase, and superoxide dismutase. The results obtained from this analysis were used to classify strains by numerical analysis based on pair-wise similarity coefficients, and further to propose the delineation of groups of strains.

On the basis of physiology two suprageneric or sub generic groups have been proposed (Lechevalier and Lechevalier 1989; Lechevalier and Lechevalier 1979). Main differences are shown in table 2.1(See table 2.1 at the end of this chapter).

The "constant" (relatively invariable for all taxa) regions of the 16S rRNA nucleotide sequences of actinomycetes are valuable for determination of the proper

placement of genera within the order Actinomycetales and for discerning the position of the order Actinomycetales in the prokaryote, while it is widely believed that the "hyper variable" regions may help in phylogenetic classification at the genus or species level (Harry *et al.* 1991; Mirza *et al.* 1992). In a different approach by Simonet *et al.* (1991) two different sets of primers were designed. One of which was a universal set designed for nine nitrogen fixing genera and the other set was specific for the *nif*H-*nif*D regions of *Frankia* spp. augmentation of different strains of *Frankia* with these two sets of primers resulted in fragments of the length specific for the genus. Similarly universal and specific primers targeted to 16S rRNA gene region also generated the fragments of the size characteristic of *Frankia* spp. and also of the closely related genus *Geodermatophilus* and were therefore presumably specific for members of the family Frankiaceae. Members of the genus *Geodermatophilus* and *Frankia* were further separated on the basis of intergenic sequence [IGS] the 16S and 23S rRNA genes by designing two additional primers. The amplification results showed that the 561bp fragment generated by the *Frankia* differed from the size of fragments generated from *Geodermatophilus* strains and the other microorganisms tested. Although this technique is quite promising, more strains have to be analyzed before general conclusion can be drawn.

2.2.2. Taxonomy of the species

The most critical part of *Frankia* taxonomy is its speciation. Main difficulties include (1) individual strains often nodulate plants from different plant orders (Benson and Silvester 1993), (2) strains from same genomic species have been isolated from members of different plant families (3) some strains failed to re-infect their source plants (Mirza *et al.* 1991) and (4) unpredictable nodulating abilities have been observed among the *Elaegnus* strains (An *et al.* 1985; Fernandez *et al.* 1989). Nevertheless, a considerable degree of host plant specificity does occur, with the result that the host plant origin and the ability to nodulate within certain host specificity groups are relevant but not determinative characteristics for strain identification or classification.

Arranging *Frankia* strains into phenotypically related groups have proven to be a difficult task. Classical physiological testing is of little use in grouping *Frankia* strains since they all grow slowly (doubling times of 15 to 48 h or more and the results often

vary depending on how long the strains are allowed to grow. Consequently, several other approaches have been taken with various degrees of success. Early attempts to correlate serological groups with host range or other phenotypic parameters were largely abandoned when such correlations proved more complex than was initially thought. (Baker *et al.* 1981; Lechevalier *et al.* 1983).

Techniques applied to resolve these problems are morphological differences, in planta sporangia formation (Normand and Lalonde 1982), phylogeny of carbohydrate uptake (Lechevalier *et al.*1983; Ganesh *et al.* 1994), utilization of various substrates and enzyme production (Shipton and Burggraaf 1982), serology (Baker *et al.*1981; Lechevalier *et al.*1983), host specificity (Bosco *et al.*1992) isozyme patterns (Maggia *et al.*1990), whole cell difference in the ribosomal sequences of genomic species of isolates of Elaeagnaceae etc.

Lalonde *et al.* proposed the acceptance of the species *F.alni* and *F.elaeagni* and created *F.alni* subsp. *pommerii* and *F.alni* subsp. *vandijkii* (Lalonde *et al.*1988). This proposal was based on the results of biochemical and physiological studies done on strains isolated mainly from members of the families Betulaceae, Myricaceae, Elaeagnaceae, and Casuarinaceae.

Fernandez *et al.* (1989) identified nine genomic species among 43 isolates examined. Three genomic species were found among the strains that infect *Alnus* species, five were found among strains that infect members of the family Elaeagnaceae, and one contained 11 strains isolated from members of the Casuarinaceae. Nine strains (four isolated from *Alnus* species, two isolated from members of the Elaeagnaceae, and three isolated from members of the Casuarinaceae) were not classified and may represent additional genomic species.

The largest group of *Alnus* infecting strains, genomic species 1, was first established as genogroup I by An *et al.* in their early DNA homology work (An *et al.*, 1985). They established genogroup I with seven isolates from *Alnus* species plus one from *Comptonia peregrina* and one from *Myrica pensylvanica*, with DNA from Arl49 (from *A. rubra*) as a reference. The coherence of this group has since been confirmed by numerous phenotypic and genetic analyses and thus has a valid claim to the species

epithet *F.alni* as described by *Lalonde et al.* (1988) with strain HFPCpI1 as the type strain.

The proposal that the subspecies *F.alni* subsp. *vandijkii* and *F.alni* subsp. *pommerii* be established has generated some confusion in the naming of strain. As Beyazova and Lechevalier (1992) have pointed out, a subspecies of *F.alni* should be *alni*. Also, strain ArgP5^{Ag} (ULQ0132105009), which is the type strain for the *F.alni* subsp. *Vandijkii* group of Lalonde *et al.* (1988), was in genomic species 3 in the analysis of Fernandez *et al.* (1989) and had only a 2% DNA homology with members of genomic species 1, which contained HFPCpI1. Therefore, the proposal that *vandijkii* be considered a subspecies of *F.alni* is problematical, since a subspecies should be genetically close to but phenotypically distinct from the type species (Wayne *et al.* 1987). Because of the results from the DNA-DNA reassociation studies, the definition of the subspecies *vandijkii* and *pommerii* needs to be reconciled with current knowledge. DNA-DNA reassociations have confirmed the diversity in the physiology, morphology, and biochemistry of Elaeagnus strains (An *et al.* 1985; Fernandez *et al.* 1989).

An *et al.* (1985) found no genogroup among 10 isolates (5 from *Elaeagnus* species, two from *Casuarina equisetifolia* and one each from *Ceanothus americanus*, *Purshia tridentate*, and *Alnus incana* subsp. *rugosa*) tested against DNA from EuI1a from *Eleagnus umbellate*) or ArI4. The 13 Elaeagnaceae strains tested by Fernandez *et al.* (1989) constituted their genomic species 4 through 8. Genomic species 4 had six members. Species 5 had three, and species 6, 7, and 8 had one each. Unfortunately, the type species proposed for *F. elaegnii*, strain SCN10a (ULQ190201001) (132), was not studied, so its relationship to other genomic species remains unknown. Strains originally isolated from members of the Casuarinaceae showed a surprising degree of similarity. Fernandez *et al.* found that out of the 11 strains tested (Fernandez *et al.*, 1989), 8 constituted genomic species 9, with percent reassociations ranging from 69 to 100%. The three strains that remained unclassified were "atypical", since they failed to reinfect members of the Elaeagnaceae.

Nazarat *et al.* (1991) developed a protocol based on the PCR amplification and sequencing of 16S ribosomal DNA sequence to measure phylogenetic relationships.

They examined nine genomic species created by Fernandez *et al*, earlier in 1989 and found that with one exception, strains belonging to same genomic species had identical sequences and that they differed from other genomic species

Another important method for understanding phylogeny of *Frankia* is restriction fragment length polymorphism (RFLP) of *nif* complexes (*nif*AB, *nif*K and *nif*H). The *nif* D-K DNA IGS region in *Frankia* spp. is larger and more variable than the *nif* H-D IGS region; thus the PCR-RFLP study of *nif* D-K IGS has proven to be more useful for distinguishing among closely related strains. Nazarat *et al.* (1989) found highly homologous results for infective isolates obtained from *Casuarina* spp. regardless of restriction enzyme used, whereas non-infective strains differed from first group and among themselves. Akimov and Dobritsa (1992) observed high level of DNA-DNA relatedness among both infective and non-infective isolates. Simonet *et al.* (1989) did RFLP analysis of 100 isolates from single alder stand using *nif*-HDK probes and they found similarity. When they used *nif*AB probes, five groups were found.

Jamann *et al.* (1993) employed PCR-RFLP technique. They amplified intergenic spacer (IGS) and a part of genes in *nif* cluster through PCR using two primers composed of sequences from conserved *nif*-D and *nif*-K regions flanking *nif* D-K IGS region. They cleaved the Amplicon with four different restriction enzymes and patterns obtained were used as fingerprints for typing *Frankia* strains. They also revealed that the DNA of *Frankia* isolates obtained from members of Elaeagnaceae and Casuarinaceae could be amplified to yield a product of about 1380bp, whereas the DNA of isolates obtained from *Alnus* spp. were amplified only weakly or were not amplified. When these products were digested with various restriction enzymes like *Hinf-I*, a characteristic pattern is obtained i.e. products digested with *Hinf-I* grouped together the entire test isolates *Mip-I* and *Scfr-I* resolved these organisms into the genomic spp. previously determined by DNA-DNA pairing.

Marry P. Lechevalier and her colleague K. Beyazova used a novel and simple method to tackle species problem. They used restriction enzymes with less number of restriction sites (rare cutters) to cut DNA into fragments of bigger molecular weights. Fragments were separated with pulse field electrophoresis. This method is called 'low

frequency restriction fragment analysis' (LFRFA). Findings of Beyazova and Lechevalier (1992) through LFRFA confirmed results of Nazarat *et al.* (1991) that type strain of *Frankia alni* subsp. *pomme* clusters at some distance from other *Frankia* genomic species isolated from *Alnus* spp. Each of these approaches has yielded phenotypic and in some cases genotypic subgroups within host specificity groups.

Not all approaches have been taken with classification in mind; some have been used primarily to assess the diversity of strains isolated from an individual plant species or of strains obtained from a limited geographical area (Benson *et al.* 1984; Bloom *et al.* 1989; Gardes and Lalonde 1987). Of the techniques used, only DNA-DNA homology and perhaps the most recently developed restriction fragment length polymorphism can unequivocally place an unknown strain within a genospecies. The other techniques yield well-defined groups independent of phylogenetic relationships, but the sensitivity of the methods remains unknown. The available biochemical, physiological, and phylogenetic information on certain well-studied groups of strains has rekindled the desire to establish *Frankia* species. To do so, it is desirable to satisfy the consensus that new genotype species be defined in phylogenetic terms on the basis of DNA sequence data (Wayne *et al.* 1987). Thus, DNA-DNA reassociation kinetics that approximate 70% or greater DNA relatedness, together with a 5°C or less δT_m between strains, would support placement of two strains in the same species, with the caveat that any genospecies so identified should not be named until it is distinguishable from another genospecies on the basis of phenotypic properties. For *Frankia* strains, sufficient information is available to support the species identification of some members of the genus.

2.3. Actinorhizal plants-The host

The plants nodulated by *Frankia* are known as actinorhizal plants (Torrey, J.G. and J.D. Tjepkema 1979.). According to Benson and Silvester (1993), there are 194 species of 24 genera, but there is no detailed account given. Till date about 288 species of 24 genera belonging to 8 families of 7 orders have been reported to have actinorhizal associations (Baker and Schwintzer 1990), some of the important *Frankia*-actinorhizal

symbiosis can be seen in (Plate I & II). Table-2.2(See table 2.2 at the end of this chapter) shows some important symbiotic associations between *Frankia* and actinorhizal genera.

Actinorhizal genera are found in ancient as well as advanced lineage. They like to grow in marginally fertile soil and can be found in most of the climatic zones inhabiting a variety of ecosystems including arctic tundra (*Dryas* species), coastal dunes *(Casuarina, Hippophae, Myrica,* and *Elaeagnus* species), riparian (*Alnus* and *Myrica* species), glacial till *(Alnus* and *Dryas* species), forest (*Alnus, Casuarina, Coriaria,* and *Shepherdia* species), chapparal and xeric (*Casuarina, Purshia, Ceanothus, Cercocarpus, Comptonia, and Cowania* species), and alpine (*Alnus* species).(Benson and Silvester 1993).

Actinorhizal plants are distributed worldwide and occupy a wide range of habitat. They are native to Pakistan, eastern Nepal, Bhutan, northern India, southwestern China, upper Myanmar and parts of Indochina. They are introduced to various countries in Africa, Central America and South-East Asia. Table 2.3 shows the distribution of native actinorhizal families and genera (See table 2.3 at the end of this chapter).

Distribution of Actinorhizal plants in India is shown in Fig.2.1 (See figure 2.1 at the end of this chapter)

2.3.1. Taxonomy of host plant

All the actinorhizal plants are trees or shrubs, except for the genus *Datisca,* which is herbaceous. Besides, all the members of a certain family may not be actinorhizal, while all the genera of the family Elaeagnaceae are actinorhizal. They cover a range of woody dicotyledons and the taxonomic relation is very less. Some species are very well adapted to flooded lands, warm arid and semiarid regions and areas of devastation (for example, rock slides). The taxonomic position of actinorhizal genera in the Magnoliopsida according to Cronquist (1988) is quite different from the position in Hutchinson's (1973) classification .Particularly, in Cronquist's classification, Rosales are not ascribed to a position anterior to the Hamamelidales and the Elaeagnaceae and Rhamnaceae are found in different orders and are shown to have diverged from the

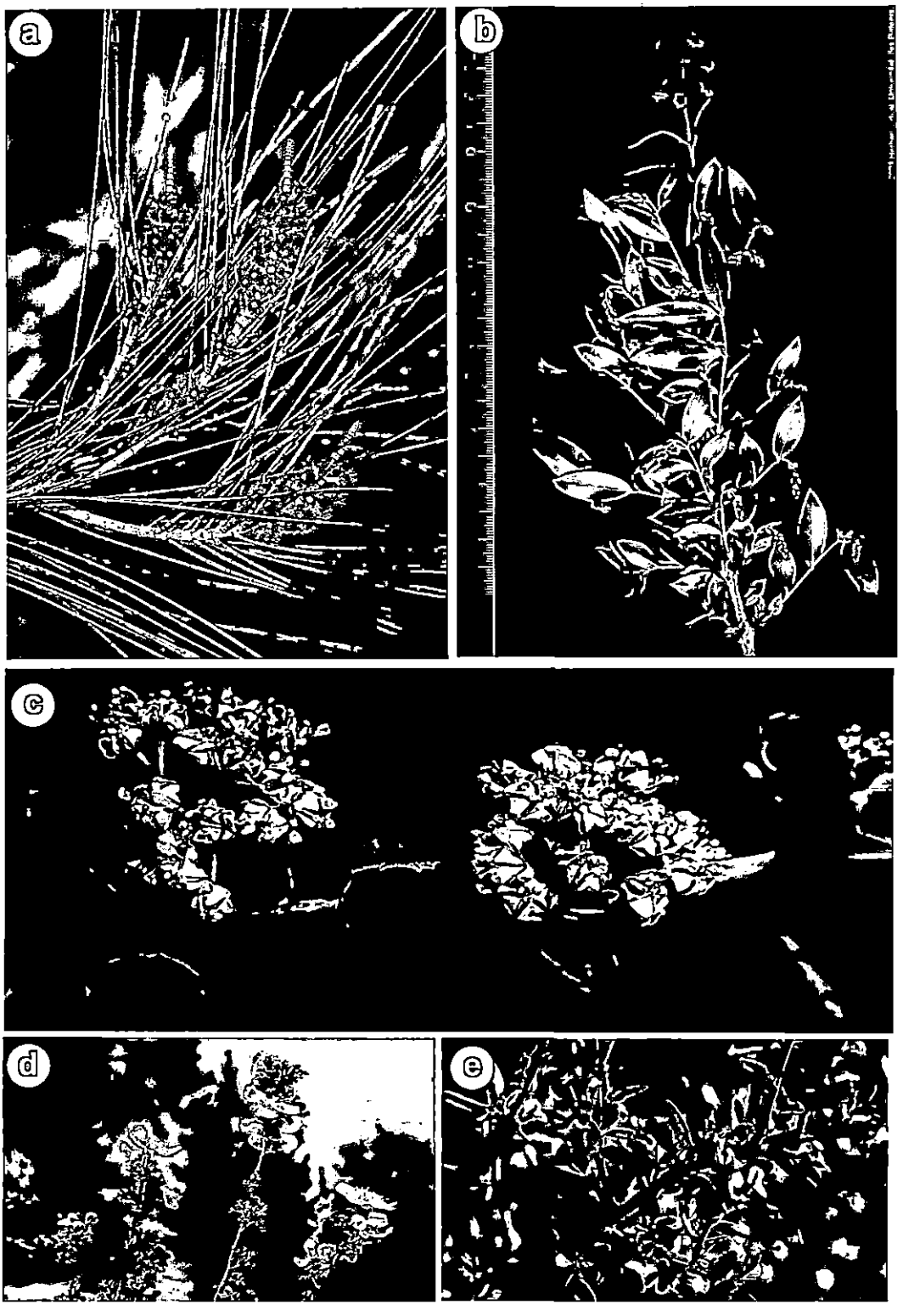

Plate I

Representatives of Actinorhizal plants : (a) A twig showing inflorescence of *Casuarina glauca* (b) A flowering twig of *Coriaria myrtifolia* L. (c) Flowering twig of *Ceanothus gloriosus* var. *Exaltatus.* (d) An inflorescence of *Pueshia stansburiana* (e) A plant of *Cercocarpus* sp.

Photo courtesy : www.ibiblio.org.; www.Herbarl Virtual, Universitat Illes Balears; www.nazflora.org.

Plate II

Representatives of Actinorhizal plants : (a) Flowers of *Cowania* sp. (b) A plant of *Cowania* sp. (c) Plant with fruiting body of *Eleagnus multiflora* (d) A plant of *Myrica gale* showing its flowers.

Photo courtesy : www.unps.org; www.floridata.com.

Rosales. Datiscaceae are found in the Violales (Dilleniidae and Casuarinales are grouped together with Fagales and Myricales in the Hamamelidae.Pollen records show, members of the orders Fagales and Myricales were the first among the actinorhizal plants to visit the earth. Rosales, Proteales and Rhamnales are relatively advanced.

The host involved in the present study is *Alnus nepalensis*, commonly called alder. The alders belong to the family Betulaceae. They are known by various Synonyms such as *Alnus mairei*, *A. Léveillé*, *Clethropsis nepalensis* Spach. The vernacular/common names of alder are Utis (Nepal); maibau (Burma); piak (India); meng-zi-qi-mu, han-dong-gua (China); tong quan su, tong qua mu, tong po mu (Viet.);Indian alder, Nepalese alder (Eng.) (Joker 2000).

Alnus prefers moist, cool climates with mean annual temperature of 13-26°C and mature trees are tolerant to frost. It 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. *Alnus* species are mostly drought tolerant 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. *Alnus* does not require high soil fertility but prefers permeable soils. It does poorly on dry, exposed ridge tops (Joker 2000).

The alder plants are deciduous or semi-deciduous trees, typically about 30 m tall and with a diameter of 60 cm but on good sites it can reach 35 m and a diameter of 2 m. Leaves are simple, alternate, 6-20 cm long, slightly serrate with prominent parallel veins. The flowers are unisexual, female and male flowers in separate inflorescences called catkins. Male catkins 10-25 cm long, are drooping, in terminal panicles. Female catkins 1-2 cm long, 3-8 together in axillary racemes. The fruiting catkins resemble cones. They are dark brown, 1.5-2 cm long, upright on short stalks, elliptical and with woody scales. The empty catkins persist on the tree. The seeds are light brown, circular, flat nut, with membranous wing, more than 2 mm across. Eight kg of catkins contain about one kg of seed. There are typically 2.3-3.5 million clean seeds/kg. (Joker 2000).

In Nepal, seed is collected between November and March depending on locality. In Yunnan (China) seed ripens in December. The catkins are collected directly from the

tree when they turn yellowish-brown and begin to open, but before the seeds have been dispersed by the wind. Catkins from previous years can persist on the tree; they are dark brown or black, contain no seeds and should be avoided. (Joker 2000).

2.4. Actinomycetes symbiosis

Frankia forms symbiotic association with botanically diverse group woody shrubs and small trees belonging to well over 200 species. Actinorhizal plants are classified in 24 genera, eight families and seven orders as mentioned in section 2.3. Generally all species belonging to these 24 genera are able to form root nodules under proper conditions. Few exceptions however exist in Rosaceae. Some of the genera with nodule bearing species, e.g. *Casuarina*, *Coriaria* and *Myrica* have an isolated taxonomic position and are classified in orders with only one genus. In other cases the taxonomic position of nodulated species may vary considerably. For example, in Fagales, only one genus, i.e. *Alnus* is nodulated, while other genera of Betulaceae and other families of this order are non-nodulated. In Rhamnales, nodulated genera are only known in only two families, viz. Elaeagnaceae and Rhamnaceae. In Elaeagnaceae nodulation is the characteristic feature of whole taxon. In Rhamnaceae, however, only three genera viz. *Ceanothus*, *Discaria* and *Colletia* have been reported to be nodulated. However, only a few species of 16 of the 58 genera of this family have been searched for nodulation (Bond 1976). Hence there remains a probability of finding a new nodulated species in this family. If nodulation were considered to be genetically determined, one would expect that genera taxonomically related to nodulated genera, also includes nodulated species.

The strong *Frankia*-actinorhizal association reveals that nodulation does occur in various taxa of host plants, which are not always taxonomically related. However, within the taxa, nodulation in general is a feature of the genus, and often even of the whole family. Therefore plant taxonomy is an important key in discovering nodulated plants. An additional key in this study is ecology. Most nodulated plants grow on nitrogen poor soils. Such conditions are favorable to either plants with nitrogen fixing root nodules, or

to plants, which grow oligotrophically. Hence the importance of systematic study of the occurrence of nitrogen fixing root nodules cannot be ruled out.

In order to determine the efficiency of nitrogen fixation Huss- Dannel, 1990 made a thorough assay on the nodule respiration, nitrogenase activity and hydrogenase activity. With one exception of the "local source" of *Frankia* from Sweden (Sellstedt and Huss-Danell 1984; Sellstedt 1989), hydrogenase activity is universally present in *Frankia* and actinorhizal nodules. Hence nitrogenase activity undoubtedly is the strong criteria in characterizing the host-*Frankia* symbiosis. Some *Frankia* strains are infective but ineffective i.e. they are capable of forming nodules but do not fix nitrogen. Ineffective nodules are typically small in size, contain relatively a few *Frankia*, and do not normally contain vesicles (Hahn *et al.* 1988; Berry and Sunell 1990). Ineffective nodules often occur because of intergeneric and interspecific incompatibilities (Weber *et al.* 1987; van Dijk *et al.* 1988); however some *Frankia* isolates form ineffective nodules even when inoculated on the same host species from which they are isolated (Hahn *et al.* 1988). Van Dijk and Sluimer-Stolk (1990) report that some ineffective *Frankia* have no nitrogenase actvity in pure culture and their DNA does not hybridize with *nif-HDK* probe. Hahn *et al.* (1989) found significant differences in the 16S rDNA sequence of an ineffective compared to an effective *Frankia* strain. In some cases effectiveness can be lost by mutations (Faure-Raynaud *et al.* 1990).

2.4.1. Role of host in symbiosis

The actinomycete symbiosis with the host plant suggests that the host cells and tissues exert a remarkable degree of control over the expression of the invading microorganism. This host control is part of the symbiotic relationship which keeps the invader under control and allows the symbiosis to work to the benefit of the host while providing the endophytic symbiont a site and substrates to function.

The nitrogen fixing effectiveness of host *Frankia* species have been studied primarily with *Casuarina* and *Alnus* species. Over three fold differences in growth and nitrogen fixation have been found for various *Casuarina-Frankia* combinations (Redell and Bowen 1985; Fleming *et al.* 1988; Redell *et al.* 1988; Sanginga *et al.* 1990), although

Rosbrook and Bowen (1987) found no significant *Frankia* strain effect. Differences in host provenance may be greater determinants of growth and nitrogen fixing effectiveness than variations among *Frankia* strains (Sougoufara *et al.* 1992). Similar differences in growth and nitrogen fixation have been found for *Alnus-Frankia* combinations (Normand and Lalonde 1982; Wheeler *et al.* 1986; Hooker and Wheeler 1987; Weber *et al.* 1989). In *Alnus-Frankia* symbiosis, greater nitrogen fixing effectiveness has been reported for Sp^- compared to sp^+ symbioses (Normand and Lalonde 1982; Wheeler *et al.* 1986), although this relationship is not always observed (Kurdali *et al.* 1990). Despite the large variation in nitrogen fixing capacity of various host-*Frankia* combinations, no clear superior host genotype or *Frankia* strain have been recognized and any positive effect of inoculation with superior *Frankia* strain may soon be overshadowed in the field by indigenous *Frankia* (Hooker and Wheeler. 1987). In *Alnus-Frankia* symbiosis, it has been shown (Hall *et al.* 1979) that an important improvement in the rate of nitrogen fixation can be gained by the selection of host plant, which is more active in photosynthesis, the key element for nitrogen fixation activity. Furthermore the susceptibility of the host plant to a *Frankia* strain is variable and consequently selectable (Tremblay *et al.* 1984).

There is remarkable difference in the rate of symbiosis in terms of ultra-structure of *Frankia* in pure culture. The host genotype is shown to control the nodule morphology. The symbiosis in *Myrica gale* and *Comptonia* may represent an intermediate situation between *Alnus* and *Casuarina*. The terminal vesicle within the root nodule may vary in shape considerably from overtly enlarged club shaped terminal endings to only slightly enlarged elongate terminal filaments. The state of vesicle envelope within these nodules has not been determined. As in *Casuarina*, the cell walls of infected *Myrica* nodules show specialization (VandenBosch and Torrey 1985) and intermediate level of haemoglobin like compound has been reported (Tjepkema 1983). Atmospheric levels of O_2 are available within the nodule to the infected cell (Tjepkema 1983) and even in water logged sites, aeration of the nodules through air passages within the nodule roots allows the nodules to undergo aerobic respiration and would necessitate some special modifications of the infected cells to achieve O_2 protection of the

nitrogenase within the *Frankia* filaments and vesicles. The interesting and peculiar orientation of the elongate vesicles in *Datisca* and *Coriaria* suggests yet another modification, which may have evolved to achieve the end of O_2 protection of the endophyte nitrogenase.

2.4.2. *Alnus-Frankia* versus legume-*Rhizobium* symbiosis

Although rhizobial symbiosis is by far the best studied of the nitrogen-fixing symbiotic associations with higher plants, many others exist. Symbiosis between woody dicotyledonous plants and the actinomycetes, *Frankia* (a filamentous prokaryote), has been reported in 21 genera covering eight different families and is believed to have a role in nitrogen-fixation comparable to that of rhizobia symbiosis.

Many similarities exist between rhizobial and the *Frankia* symbiosis. The mode of symbiosis and root nodule induction procedure of *Frankia* and *Rhizobium* are more or less similar, both lead to the formation of nodules on the roots of the host plant in which nitrogen fixation occurs. In both cases there is a complementarily with some of the *nif* gene sequences of *K. pneumoniae* and a number of associated proteins are related. However, *Frankia* doesn't invade the host cell and is not therefore surrounded by a poribacterial membrane. Instead it is encapsulated in carbohydrate material derived from the host plant. This may not be distinctive since legume (*Andria* sp.) has shown that similar encapsulation occurs. Other evidences suggest that the *Frankia* symbiosis involves hemoglobin similar to that found in legumes and that the mechanism regulating expression of nitrogenase in presence of oxygen and ammonium are very similar in both *Rhizobium* and *Frankia*. They differ markedly in development in morphology and development. Infected zone of legume nodule is central and is normally contending within an endodermis and an inner cortical layer of tightly packed cells. Actinorhizal nodules normally have a central stele that has infected tissue adjacent to it or around it (Benson and Silvester 1993).

2.4.3. Importance of Alder-*Frankia* symbiosis

Study of the *Frankia* actinorhizal symbiosis is important for several reasons, as a pioneer, nitrogen-fixing species alders are suitable for soil improvement and rehabilitation of degraded lands, including the use of actinorhizal shrubs and trees for reforestation and reclamation of depauperate, nitrogen limiting soils. Seeds have been broadcast to stabilize landslides (Gordon and Wheeler 1983). In Burma, it has been used with success to reforest abandoned taungya areas. In agro- forestry systems, it can be interplanted with a number of crops. The wood is an important source of firewood and charcoal.

For example, as more and more land, especially in the tropics, becomes deforested, the utilization of fast growing, nitrogen fixing trees for reforestation purposes becomes critical. According to the Office of the Technology Assessment (OTA) (1984), about 11.3 million hectares of what remains of tropical forests are destroyed annually. In addition many non-leguminous, nitrogen-fixing plants are colonizers of poor soils. After the Pleistocene glaciation's, plants such *as Alnus, Dryas, Elaeagnus, Hippophae*, and *Shepherdia* played an important role in soil reconstruction. (Lawrence *et al.* 1967). Currently their role in nitrogen fixation is recognised as being at least comparable to that *Rhizobium*-legume symbiosis and attempts are being made to utilize actinorhizal trees in reforestation and soil reclamation in developing countries. Besides the practical applications of actinorhizal plants to situations described above, the study of the symbiosis offers much interest to those investigating the basic biology of nitrogen fixing symbiosis. There are a number of parallels, developmental and genetic, between the *Frankia*-nonlegume symbiosis and the *Rhizobium*-legume association, which make the former interesting to study in terms of molecular biology. Some of these similarities are striking. For example, it has been known for some time that the *nif* genes of *Klebsiella pneumoniae* hybridize with DNA sequences from *Frankia* as well as from *Rhizobium*. Both the *Rhizobium*-legume and *Frankia* -nonlegume symbiotic associations lead to the formation of root nodules in which the microbes fix atmospheric nitrogen to ammonia. However, we must keep in mind not only the similarities, but also the differences, between the two types of symbiosis.

In exchange for a water and nutrient supply, the bacteria enable alders to fix much needed atmospheric nitrogen, which in turn enriches the surrounding soils. This increases the fertility of the soil for many species and makes alders as important species in ecological successions, land reclamation and remediation. Thus, *Frankia* have a direct and important impact on the environment and it makes the alders as an important and unique species in the world.

2.5. *Frankia* Physiology

The hydrogen metabolism of *Frankia* seems to be an interesting physiological characteristic. All nitrogen-fixing organisms evolve hydrogen from the nitrogen-fixing enzyme, nitrogenase, when nitrogen is reduced to ammonia. This means that some of the energy used in the nitrogenase reaction is wasted on nitrogen evolution. Root nodules of many legumes (*Rhizobium* symbioses) evolve considerable amounts of hydrogen with as much as 30%-60% of the electron flow through lost as hydrogen (Schubert and Evans 1976) .In contrast, *Frankia* symbioses were claimed to have little or no net hydrogen evolution (Moore 1964; Schubert & Evans 1976, Roelofsen & Akkermans 1979). In some strains of *Rhizobium* and *Frankia* hydrogenase is present (Roelofsen and Akkermans 1979; Benson *et al.* 1980) and recycles (part of) the hydrogen evolved from nitrogenase. Hereby some energy is regained and no or little net hydrogen evolution is measured.

Recently lack of hydrogenase activity was reported from a symbiosis between *Frankia* and *Alnus incana* (Sellstedt and Huss-Danell 1984). This *Frankia* strain makes it possible to study the importance of differences in hydrogen metabolism for growth and nitrogen accumulation in *Alnus*. The purpose of the present study was, therefore, to compare symbioses between one clone of *Alnus incana* and different strains of *Frankia* with respect to nitrogen fixation and growth of alders in relation to hydrogen metabolism of the *Frankia* strains

2.6. *Frankia* in pure culture

2.6.1. Morphology of *Frankia* in pure culture

Invitro studies of *Frankia* were possible only after the isolation and subsequent axenic culture on yeast extract medium. But improvements in the composition of culture media now permitted *Frankia* to be grown *invitro* much faster than earlier used yeast-extract medium (Lalonde and Calvert, 1979). In their classical reviews Newcomb and Wood (1987) and Benson and Silvester (1993) gave vivid descriptions of structure and ultra structure of *Frankia* cells. Benson and Silvester (1993) also discussed the difficulties of its study with electron microscope because of the delicate nature of some components of cells.

In pure culture *Frankia* behave as micro-aerophilic and mesophilic microorganism (Burggraaf and Shipton, 1982). It forms dense mat of anastomosing hyphae with sporangia developing terminally and at intercalary positions when grows *invitro* (Newcomb and Wood, 1987). Unlike other symbiotic nitrogen-fixing bacteria, *Frankia* is multicellular and differentiated. During the life cycle, vegetative hyphae, vesicles and sporangia develop in response to partially defined conditions (nitrogen limited). *Frankia* strains are characterized by the presence of vesicles and hyphae bearing large sporangia when cultivated under proper conditions. These spherical structures have not yet been found in other kind of actinomycetes and can be utilized as the feature of the genus *Frankia*. However, spore formation and induction of vesicles are largely dependent on the culture conditions. This makes the identification of the strain difficult. In many strains the production of vesicle is suppressed by the presence of combined nitrogen in the medium, i.e. conditions which also repress the nitrogenase.

2.6.1.1. Hyphae

Frankia strains are readily recognized by their appearance in liquid culture. They form extensive hyphae and spores on multilocular sporangia located either terminally or in intercalary position on the hyphae. Aerial hyphae are not produced on solid media. Free-living cells under the light microscope show branched sepate hyphae ranging in

width from 0.5 – 1.5 μm in diameter. The hyphal cell wall is composed of two layers of electron dense material, a base layer and an outer layer. Base layer gives rise to cross walls. Internally hyphal cells contain numerous rosette- shaped granules, which are presumed to be glycogen and lipid droplets. An extra cellular multilayered envelope was first identified in hyphae of free-living *Frankia alni* HFPCpI1 (Newcomb *et al.* 1979).

2.6.1.2. Sporangia

Frankia sporangia are observed in the cortical tissue of some actinorhizal nodules. Sporangia in the nodule structurally resemble those formed in free-living culture, and are located both within infected cortical cells and in intercellular spaces of some hosts. In *Alnus*, *Myrica* and *Comptonia*, differentiation of sporangia coincides with vesicle senescence (VandenBosch and Torrey 1984). Host cytoplasm degenerates in cells where sporangia are present.

Frankia sporulate spontaneously in submerged culture, forming multilocular sporangia, which are round, cylindrical or irregular in shape (Newcomb *et al.* 1979). Sporangia develop as terminal or intercalary structures and remain filled with thick walled, refractile and non-motile spores that are roughly geometrical in shape (Newcomb and Wood, 1987). The mature spores show evenly dispersed, but the tubules, which are such a prominent feature of hyphae, are not present in the developed spores (Lancelle *et al.* 1985). Cytoplasm Spores are surrounded by a lamina and their cytoplasmic content is similar to that of the hyphae. The tubules, which are present in the laminate envelopes of hyphae, are absent in the sporangia (Lancelle *et al.* 1985). Sporulation in nodules is well documented only for *Alnus* and *Myrica* species and even in these, is sporadic and somewhat site specific (Torrey 1987). Sporulation in nodules of other genera is not well documented. Strains, which release spores, show good germination in culture (Tzean *et al.* 1989) and, coincidentally perhaps, also show low levels of sporulation in host plants (Racette *et al.* 1991)

Although virtually all *Frankia* can be induced to form sporangia in pure culture, this is not necessarily the case in planta. Van Dijk and Merkus, (1976) observed that

Frankia within actinorhizal root nodules either forms many sporangia (Sp+) or no sporangia (Sp-). Smolander and Sundman (1987) proposed an intermediate class for nodules devoid of sporangia, however, Schwintzer (1990) suggests that intermediate nodules are better classified as Sp-.The spore type of actinorhizal nodules is of interest for at least three reasons: (1) It is doubtful if any *Frankia* have been isolated from Sp+ nodules, (2) Interesting and complex ecological relationships have been observed between Sp+ and Sp- strains of *Alnus* sp. and *Myrica* gale (Weber 1986; Holman and Schwintzer 1987; Kashanski and Schwintzer 1987; Smolander and Sundman 1987), and (3) There is some evidence that Sp+ and Sp- nodules differ in both absolute and relative nitrogenase efficiency

2.6.1.3. Vesicles

Frankia produces vesicles *in vivo* (exception: *Casuarina*). Most isolates of *Frankia* differentiate vesicles at the end of hyphal tips under conditions of ammonia starvation (Fontaine *et al.* 1984). In host plants, *Frankia* vesicles assume various shapes. In nodules of *Alnus* and *Elaeagnus* the spherical vesicles are arrayed around the periphery of infected cortical cells of the nodule lobes (Lalonde and Knowles 1975; Baker *et al.* 1980). Vesicles are internally septate, stiptate and circular or cone like in shape; approximately 1.5-2.0µm in diameter. Vesicles are mainly of three types: (1) Spherical vesicles are found in *Alnus* and *Elaeagnus* nodules, (2) Members of Rosaceae i.e. *Cercocarpus* and *Dryas* species have nonseptate and elliptical vesicle (nitrogenase protection mechanism is unclear in this case), (3) Club shaped hyphal endings which can be called as vesicles are found in the species of *Casuarina* and *Comptonia* (Newcomb and Wood 1987).

Vesicles originate as basally septate, stiptate swellings of hyphae or short side branches, termed pro-vesicles. The densely cytoplasmic pro-vesicles often show evidence of internal septation. The most important physiological function of vesicles is to shelter nitrogenase, the key enzyme involved in nitrogen fixation. As in all prokaryotes the enzyme nitrogenase in *Frankia* is oxygen labile (Benson *et al.* 1979). Within the vesicle, nitrogenase is protected from deleterious effects of oxygen. Special protection of

the enzyme is provided in a variety of ways. One hypothesis is that the multilaminate vesicle envelope developed in response to increase in ambient pO_2 provides a physical barrier to inward diffusion of oxygen, thereby protecting nitrogenase within the vesicle from denaturation by O_2.

Vesicles are also found in planta, where their shape or presence can be determined by the host (Baker and Mullin 1992). Vesicles are notably absent from nodules of species of *Casuarina* and *Allocasuarina*, yet isolates from these nodules differentiate vesicles in culture (Tjepkema and Murry 1989). Since in *Casuarina* nitrogen fixation occurs in absence of *Frankia* vesicles, a specialized host cell wall with hydrophobic properties, which could function as oxygen diffusion barrier (Berg and McDowell 1988), is thought to be the structural barrier for nitrogenase.

Vesicles have been proposed as the sites of nitrogen fixation (Noridge and Benson 1986; Meesters 1987). In most *Frankia* strains vesicles are formed only in response to nitrogen limitation (Fontaine *et al.* 1984; Murry *et al.* 1984; Tjepkema *et al.* 1980), but some strains can produce vesicles even on nitrogen rich media (St.Laurent and Lalonde 1987; Meesters 1987; Tisa *et al.* 1983).

2.7. Nodule development

Actinorhizal root nodules are established through a series of interactions between *Frankia* and host root cells. The nodule is system of structurally and developmentally modified lateral roots. The structural changes observed in the development of nodules reflect the molecular events in which normal patterns of plant development are altered.

2.7.1. Nodule morphology

Actinorhizal root nodules are perennial, coralloid structures consisting of multiple nodule lobes. Field collected nodules 3-5 cm. In diameter are not uncommon. Nodules may consist of discrete lobes, as in *Myrica* or *Ceanothus*, or the lobes may be densely packed as in *Alnus*. Each nodule lobe is a modified lateral root. The nodule meristem gives rise to mature tissues, including a vascular cylinder, cortical tissue and superficial

periderm. In most cases, nodule lobe growth is determinate. *Frankia* infects only cells of nodule cortex.

In several actinorhizal hosts, a more indeterminate pattern of growth is reflected n the formation of nodule roots from the apex of mature nodule lobes. In *Myrica* and *Casuarina*, nodule roots, which are negatively geotropic, develop from most nodule lobes. Nodule roots are devoid of *Frankia* hyphae and vesicles, and so do not contribute to nitrogen fixation. Nodule roots often contain large air spaces, an anatomical feature that facilitates oxygen diffusion.

2.7.2. Conditions for infection

.The infection process begins in the rhizosphere. Conditions necessary for colonization and initial binding of *Frankia* to the host root have not been entirely elucidated. *Frankia* hyphae are embedded within a mucilage layer at the root hair or epidermal surface. This mucilage layer is evidently secreted by the root hairs (Berry and Torrey 1983), although the root cap also produces mucilage. Elements of the host-derived matrix may participate in attachment phenomenon.

In case of root hair infection, though one infection is enough to bring about nodulation, frequency of nodule formation is directly proportional to amount of inocula (Newcomb and Wood 1987). More than one strain has been isolated from single nodule. It implies that more than one infected root hair sometimes forms one root nodule

As long as the conditions favorable for root growth are present, environmental factors such as pH or nutrient availability do not appear to limit infection and nodulation, at least for *Frankia* strains isolated to date. "Helper" microorganisms for example, *Pseudomonas* sp., improve nodulation of both *Alnus* and *Casuarina* under controlled growth conditions (Knowlton *et al.* 1980). The effect of these organisms appear to be two fold, first in including root hair deformation, which may involve altered cell wall deposition, and second in increasing the production of root hair extra cellular matrix. Even under favorable rhizosphere conditions, infection is not assured. Compatibility factors, about which little is known influence the potential for successful infection and nodulation.

2.7.3. Infection Pathways

Frankia infects its host plant in two different ways. In case of *Alnus, Casuarina Comptonia, Myrica* etc., infection takes place through root hair deformation (Berry and Torrey 1983; Callaham *et al.* 1979; Torrey 1976), while for other hosts like *Ceanothus, Elaeagnus, Shepherdia,* etc.; early colonization of root tissues by *Frankia* is entirely intercellular (Miller and Baker 1986; Racette and Torrey 1989). Despite these differences in early stages of cellular interactions between host and microsymbiont, eventually *Frankia* penetrate host cell walls in developing nodule lobes and differentiate within the cells. The same *Frankia* strain can, in some instances, infect different hosts by different pathways (Miller and Baker 1986; Racette and Torrey 1989). These observations indicate a common genetic basis for infection.

2.7.4. Infection Process

2.7.4.1. Root hair deformation

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

Root hair infection has been described in *Myrica, Comptonia, Alnus and Casuarina* (Torrey 1976; Berry *et al.* 1986). This process involves excessive changes in host cells beginning with wall deformation of expanding root hairs. Growing root hairs are perturbation by rhizosphere bacteria, especially by *Frankia.* Responding to unknown signals, growing root hairs initiate multiple tips, and tip growth is modified so those branches may bulge and wrap around each other. Branching root hairs may be few in numbers, as in *Comptonia* (Callaham and Torrey 1977), or extensive, as in *Alnus* (Berry and Torrey 1983). Cell walls of deformed root hairs fluoresce yellow-orange after

staining with acridine-orange, indicating a polyanionic component not evident in normal root hairs.

Even though all root hairs may get deformed in response to the presence of *Frankia* in the rhizosphere, only one or, in some hosts, a few root hairs become infected. *Frankia* hyphae associated with deformed root hairs penetrate at the site of folding of the root hair cell wall.

At the site of wall penetration, infection hyphae are continuous with *Frankia* in the rhizosphere. Characteristic changes occur in patterns of host wall deposition, as observed in *Alnus rubra* (Inner wall layers at the site of infection resemble transfer cell walls (Berry *et al.* 1986). Within the infected hair, the microsymbiont may branch early in infection. Whether branched or unbranched, the microsymbiont become surrounded with host plasmalemma and by host derived wall material that is continuous with the wall accumulation at the site of penetration.

After *Frankia* infection, root hairs give the appearance of high metabolic activity, while uninfected hairs degenerate, infected hairs retain cytoplasmic contents, more so at the base, with numerous ribosome's and the organelles, including a nucleus, Golgi bodies, endoplasmic reticulum, and large plastids. Vacuoles are relatively small. Mitochondria are often numerous, particularly in the vicinity of *Frankia* hyphae within the cell.

2.7.4.2. Prenodule growth and infection

With the ingression of the microsymbiont, increased mitotic activity occurs in root cortex in proximity to root hair infections. Sustained cell divisions and expansions beneath the infected hair gives rise to a region called prenodule. Externally, the prenodule is recognizable, as a slight swelling of the host root, apparent before nodule lobe primordia have emerged. Encapsulated hyphae within the root hair advance into the prenodule by penetrating the cell wall at the base of the root hair and continued cell-to-cell passage. *Frankia* further proliferates in group of expanding cells in prenodule. These cells undergo continuous hypertrophy and contain dense cytoplasm with numerous small vacuoles, Golgi bodies, mitochondria, and plastids, lacking starch grains. Portions of the

cell walls of these recently expanded cells are relatively thin. *Frankia* appears to penetrate through these thin walled regions; cell-to-cell passage does not appear to take place via plasmodesmata.

2.7.4.3. Intercellular colonization

In cases reported to date, *Frankia* appears to transit from the root cortex into the developing nodule lobe primodium through cortical cells near the base of the lobe primordium. Further progress of the hyphae within the nodule lobe occurs either intercellularly or intracellularly depending on the mode of infection. In *Ceanothus*, for the most part, hyphae do not pass from cell to cell directly (Strand and Laetsch 1977), while in *Alnus*, *Casuarina*, and *Myrica*, hyphae are clearly continuous with several cells in a file or group.

Once within a nodule cortical cell, *Frankia* hyphae branch and proliferate. As the host cell grow and differentiate it deposits encapsulating wall material, as well as plasma membrane around the hyphae. *Frankia* utilizes the encapsulation material as a metabolic substrate; since it is reported to possess pectinolytic and cellulolytic activities (Safo-Sampah and Torrey 1988; Seguin and Lalonde 1989). In all of the actinorhizal genera examined to date, the *Frankia* hyphae and vesicles always remains surrounded by the host membrane and some encapsulating wall material.

The initial intercellular colonization by *Frankia* occurs in mature root cortical tissue, while at the same time nodule lobe primodium is initiated in the pericycle. Neither cell division nor hypertrophy of root cortical cells characteristic of typical prenodules is observed in *Elaeagnus*. Ultimately hyphae penetrate and invade host cells in the developing nodule lobe. In *Ceanothus* (Rhamnaceae), an intermediate situation exists in which initial invasion of the host tissue is via intercellular colonization, as in *Elaeagnus*.

2.8. Regulation of infection and nodule development

Although the processes of infection and nodule formation are complex, there are certain key molecular and cellular phenomena that are likely to be pivotal for regulation.

We presently know very little about the molecular levels of regulation. Cellular aspects of *Frankia* symbiosis that may determine developmental patterns includes the initial contact between *Frankia* and the host root cells, cell division in the host cortex, expansion of the host cell and deposition of the modified cell walls.

2.8.1. Regulation of early infections

Root exudates are one category of signaling molecule generated by the host that could regulate *Frankia* genes concerned with nodulation. Such an interaction has been demonstrated conclusively to occur in legume-Rhizobium symbiosis where specific phenolic compounds secreted in host root exudates regulate the promoters of bacterial nodulation genes. Specific phenolics, or some other regulatory signal, might also mediate compatibility between *Frankia* and actinorhizal hosts. A host exudate factor is probably involved in regulating aspects of early nodulation.

Extra cellular matrices such as mucilage, or the host derived encapsulation layer, may be important in regulating infection. Mucilage secreted by host, both in epidermal surfaces and within intercellular spaces, may facilitate bacterial colonization; *Frankia* and other microorganisms have been shown to adhere to mucilage at the root hair surface (Berry& Torrey 1983). Binding of *Frankia* to the host cell exterior could directly trigger host responses, initially at the surface and secondarily within the host cell. Additionally, the polyanionic nature of these matrices may provide a medium for flux of cations, for example, calcium, which can affect a variety of cellular phenomena.

Extra cellular polysaccharides of host origin may provide a substrate for *Frankia* enzyme activity. Seguin and lalonde (1989) observed pectinolytic activity in culture and reported positive hybridization between *Frankia* DNA and genes coding for pectate lyase. These result suggest that *Frankia* might degrade pectic substances within nodule tissues. Resulting oligosaccharides might mediate compatibility in actinorhizal associations.

2.8.2. Control of cell division centers and cell expansion

An early consequence of *Frankia* inoculation is a localized renewal of mitotic activity in cortical tissues near sites of *Frankia* ingress. Concomitantly, lateral root primordia are initiated *de novo* in the infected zone. Such stimulation of meristematic centers during early infection suggests a regulatory role for plant growth substances. Elevated levels of cytokinins, gibberellins, and auxins in root nodules have been reported providing further circumstantial evidence for the involvement of plant growth regulators in nodule development. Auxins and cytokinins have been shown to be secreted by *Frankia* (Berry *et al.*, 1989) and may also be involved in the hypertrophy of infected cells. Another possibility is that localized cell divisions or cell hypertrophy; result indirectly from a signal from *Frankia* that triggers a host derived plant growth regulator.

2.8.3. The host cell wall and *Frankia* penetration

The host cell wall is a physical barrier to microbial invasion. Penetration of host cell wall by *Frankia* may involve wall- loosening, changes in wall structure, or wall degradation. Wall degrading enzymes produced by *Frankia* could soften or dissolve host walls. *Frankia* appears to have pectate lyase gene sequences (Seguin and Lalonde, 1989). Cellulase activity has been demonstrated in *Frankia* as well (Safo-Sampah and Torrey, 1988). Endogenous pectinolytic activity is important in fruit ripening and other developmental processes and could also be involved in wall hydrolysis during nodule development. Several structural observations suggest that new wall deposition be closely linked to successful infection. *Frankia* infects host cells during cell expansion, when primary wall is deposited, both in tip growing root hairs and in promeristematic derivatives in the nodule lobe. Wall encapsulation material is deposited around the invading and proliferating hyphae. Many of the distinctive structural features of newly infected cells, including hypertrophy, the presence of numerous Golgi bodies, Golgi derived vesicles, and microtubular arrays, indicate that cell wall synthesis and deposition are primary activities at this stage.

2.9. Phylogenetic studies on actinorhizal plants and *Frankia*

Extensive studies on symbiotic nitrogen fixing associations have shown that unlike leguminous plants which form symbiotic association with rhizobia, the actinorhizal plants are considered to be polyphyletic in their origin. On the other hand, *Frankia*-the microsymbionts forming symbiotic association with these actinorhizal plants have been assumed to be phylogenetically homologous group on the basis of a variety of their morphological, biochemical, phenotypical and nitrogen fixing capacity. (Wall 2000).

A comparison of sequences and catalogs of certain regions of 16S rRNAs by Stackebrandt and his associates showed that two type-B *Frankiae* could be shown to belong to one of the major sub-lines of descent to members of actinomycete of genus *Geodermatophilus*. The 16S rRNA sequences of type-A strains have yet to be determined. Sequencing of the rRNA genes of a single *Frankia* strain showed that the 5' to 3' order is 16S-23S-5S, which is typical of bacterial in general.

A study of the phylogeny of seed plants, based on *rbcL* gene sequences, revealed that all nitrogen fixing and nodulated plants cluster in the Rosid I lineage of the angiosperms. This result suggests that the predisposition to develop nitrogen-fixing nodules of any type arose only once during the evolution of the angiosperm (Doyle 1998). Nodulated plants within the Rosid I clade can be grouped into four major lineages: three of them include actinorhizal plants (Soltis *et al.* 1995). One includes the Hamamelid families, Myricaceae, Betulaceae, and Casuarinaceae, whereas a second includes the Rosid families Elaeagnaceae, Rhamna-ceae, and Rosaceae, as well as the *Bradyrhizobium*-infected *Parasponia* (Ulmaceae). Coriariaceae and Datiscaceae define the third line of actinorhizal plants. The fourth line of nodulated plants includes the rhizobia-infected legumes of the Fabaceae. Morphologic and anatomic features of actinorhizal nodules correlate with a more detailed analysis of *rbcL* grouping (Swensen and Mullin 1997). To date, four clades of actinorhizal plants have been defined. One of the above-mentioned groups of Rosid families is divided into two sub-clades: one including Elaeagnaceae and Rhamnaceae, and the second defined by the Rosaceae (See Figure.2.2 at the end of this chapter). Fossil records and the geographical distribution of

actinorhizal species give extra support to these groupings (Benson and Clawson 2000). Phylogenetic studies on *Frankia* have focused mainly on 16S RNA gene sequences (Benson *et al.* 1996; Jeong *et al.* 1999; Normand *et al.* 1996; Ritchie and Myrold 1999). Similar results have been obtained with *nifD* gene sequences (Normand et al.1992), and recently confirmed using *recA* and *glnII* sequences (Cournoyer and Lavire 1999). In all these studies, there has been difficulty with isolating *Frankia* from root nodules. This problem has been partially overcome by direct amplification of total nodule DNA, using specifically designed primers for *Frankia.* Consensus phylogenetic trees generated from 16S rDNA sequences consistently yield three major groups of *Frankia,* and a fourth "*Frankia*–like" clade of Nod. /Fix. actinomycetes (See Figure2.2 at the end of this chapter). Subgroups can be found, although these are not statistically well supported. There are many well-known isolates included in groups I and II, whereas no one isolate has been obtained from group III, which is defined only on the basis of analysis of nodule-extracted DNA. Physiologically, at least, the absence of a septum in vesicles of nodules of host plants infected with group III *Frankia* agrees with the proposed division. Although the phylogenies of the microsymbiont are not congruent with the four host clades, a close relationship exists between the plant and bacterial groups. Further analysis shows that the plant clades diverged earlier than the *Frankia* clades, suggesting that the *Frankia*-actinorhizal symbiosis evolved independently, at least three or four times, rather than co-evolving from an ancestral symbioses (Benson and Clawson 2000; Jeong *et al.* 1999; Swensen 1996). Nevertheless, once the symbiosis was established, the plants or *Frankia* were retained within certain taxonomic groups, with limited lateral transfer and probable co-evolution from that point onwards (Simonet *et al.* 1998). These analyses as a whole reinforce a model for host preferences of specificity in actinorhizal symbioses. (Wall 2000).

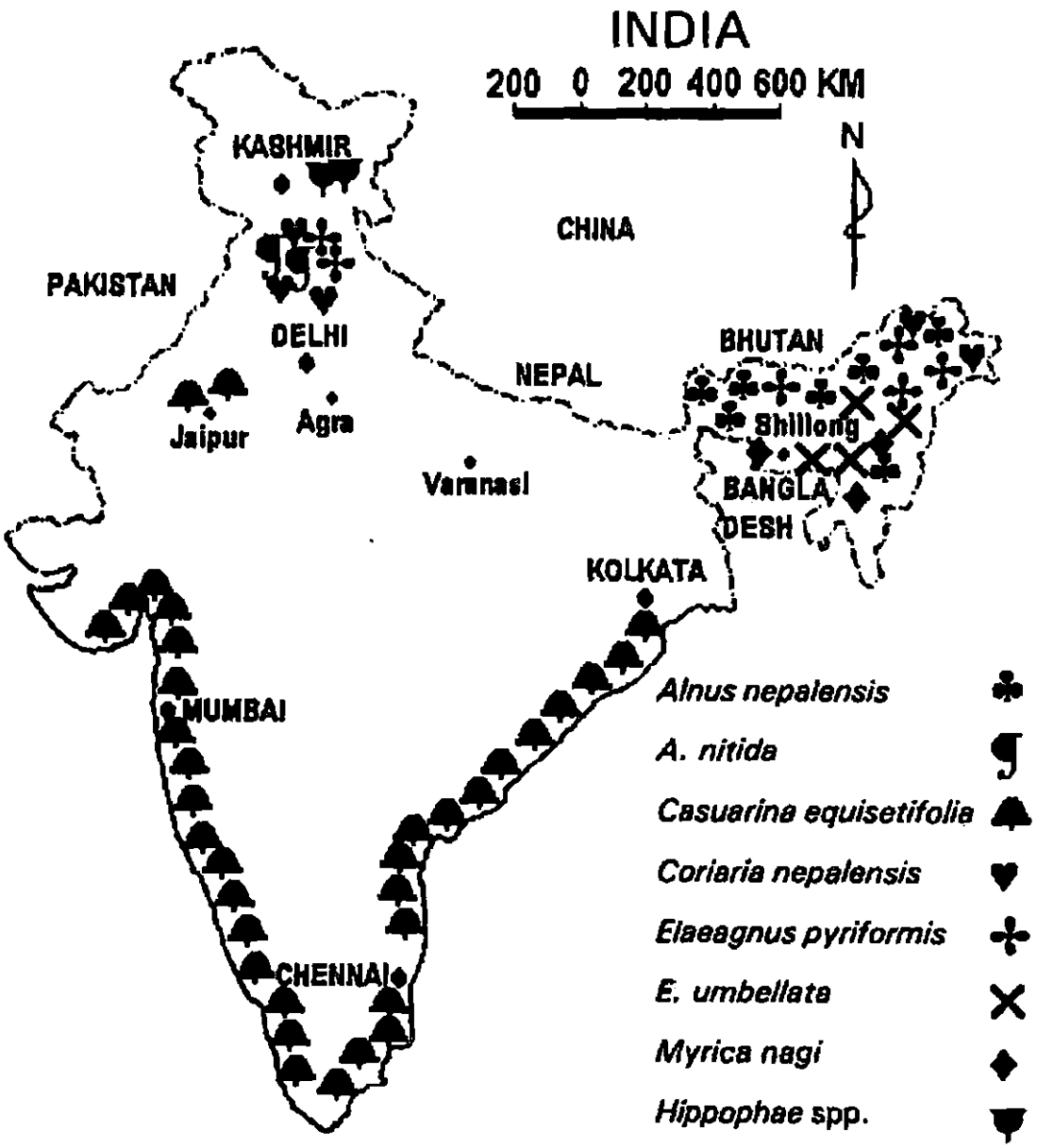

Figure 2.1: Distribution of actinorhizal plants in India

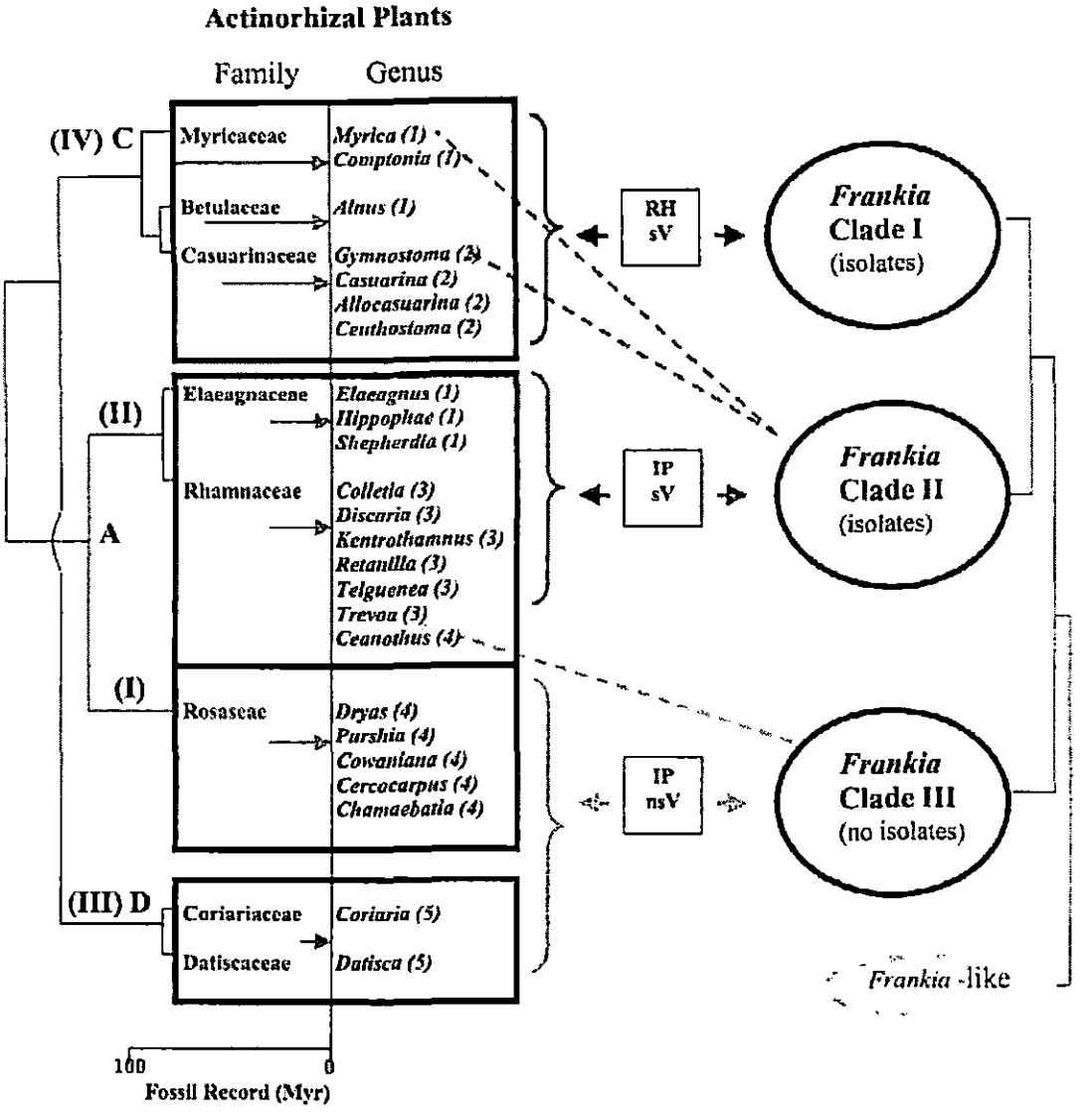

FIGURE 2.2: Phylogenetic grouping of actinorhizal plants and *Frankia*. Number between brackets of plant genus indicates native geographical distribution *(1)* to most continents, *(2)* to Australia and western Pacific, *(3)* to South America and southern New Zealand, *(4)* western North America, *(5)* disjunct distribution in northern and southern temperate zones. *RH,* root hair infection; *IP,* intercellular penetration; *sV,* septated vesicles in nodule; *ns* V, nonseptated vesicles in nodule. Based on Benson and Clawson (2000); Jeong *et al.*, (1999); Huss-Danell (1997). Groups I–IV proposed by Soltis *et al.* (1995); Clades A–D proposed by Swensen and Mullin (1977). Adapted from L.G.Wall (2000).

TABLE: 2.1 Two types of Frankial strains on the basis of physiological difference:

Type A	**Type B**
They are physiologically active.	They are physiologically inactive.
They grow relatively rapidly.	They grow very slowly.
They are aerobic and can be maintained on slants.	They are strictly microaerophilic and cannot be maintained on slants.
They do not reinfect host plant from which they are isolated, at least in the experimental conditions.	They can infect the host plant from which they are taken.
They utilize a variety of monosaccharides and disaccharides with or without acid production.	They cannot utilize carbohydrates, proteins, or starch. The preferred carbon sources are organic acids or between compounds.
Many of them produce hydrolytic enzymes, such as pectinases, cellulases, amylases, and proteases.	No such reports.

Compiled from Lechevalier (1994).

TABLE 2.2: Frankial isolates in symbiotic association with Actinorhizal plant genera

ORDER	FAMILY	GENUS	NUMBER OF SPECIES	ISOLATES
Casuarinales	Casuarinaceae	*Allocasuarina*	54	+,I,E
		Casuarina	16	+,I,E
		Ceuthostoma	2	-
		Gymnostoma	18	+,I,E
Fagales	Betulaceae	*Alnus*	47	+,I,E
Myricales	Myricaceae	*Comptonia*	1	+,I,E
		Myrica	28	+,I,E
Protales	Elaeagnaceae	*Elaeagnus*	38	+,I,E
		Hippophae	2	+,I,E
		Shepherdia	2	+,I,E
Ranunculales	Coriariaceae	*Coriaria*	16	+,N
Rhamnales	Rhamnaceae	*Ceanothus*	31	+,N
		Colletia	4	+,N
		Discaria	5	+,N
		Kentrothamnus	1	-
		Retanilla	2	+,N
		Taalguenea	1	+,?
		Trevoa	2	+,N
Rosales	Rosaceae	*Cercocarpus*	4	+,N
		Chamaebatia	1	-
		Cowania	1	+,?
		Drayas	3	-
		Purshia	2	+,N
Violales	Datiscaceae	*Datisca*	2	+,N

Symbols: (-)Isolates not reported; (+)Isolates obtained; (I)Infective; (N)Non-infective; (E)Effective in fixing Nitrogen; (?)Ineffectiveness unknown or unreported. Compiled from: Bond (1983), Torrey & Berg (1988), Newcomb & Wood (1987), Baker & Schwintzer (1990) and Benson & Silvester (1993).

TABLE 2.3: Continent wise distribution of native actinorhizal families and genera.

Continent	Family	*Genus*
North America	Betulaceae	*Alnus*
	Coriariaceae	*Coriaria*
	Datiscaceae	*Datisca*
	Elaeagnaceae	*Elaeagnus*
		Shepherdia
	Myricaceae	*Myrica*
		Comptonia
	Rhamnaceae	*Ceanothus*
	Rosaceae	*Cercocarpus*
		Chamaebatia
		Cowania
		Dryas
		Purshia
South America	Betulaceae	*Alnus*
	Coriariaceae	*Coriaria*
	Myricaceae	*Myrica*
	Rhamnaceae	*Adolphia*
		Colletia
		Discaria
		Kentrothamnus
		Retanilla
		Talguenea
		Trevoa
Africa	Myricaceae	*Myrica*
Eurasia	Betulaceae	*Alnus*
	Coriariaceae	*Coriaria*
	Datiscaceae	*Datisca*
	Elaeagnaceae	*Elaeagnus*
		Hippophae
	Myricaceae	*Myrica*
	Rosaceae	*Dryas*
Australia and Oceania	Casuarinaceae	*Allocasuarina*
		Casuarina
		Ceuthostoma
		Gymnostoma
	Coriariaceae	*Coriaria*
	Myricaceae	*Myrica*
	Rhamnaceae	*Discaria*

Adapted from Baker (1988) and Baker and Schwintzer (1990) and update based on Cruz-Cisneros and Valdes (1991).