

SAPROPHYTIC GROWTH AND FATE OF *FRANKIA* STRAINS IN SOIL
DISSERTATION

Presented to the Graduate Council of
Texas State University-San Marcos
in Partial Fulfillment
of the Requirements

for the Degree

Doctorate of PHILOSOPHY

by

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San Marcos, Texas
August 2009

SAPROPHYTIC GROWTH AND FATE OF *FRANKIA* STRAINS IN SOIL

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ACKNOWLEDGEMENTS

First of all I would like to thank my research advisor Dr. Dittmar Hahn for his constant guidance, kind supervision and valuable help at every step of my Ph.D. study. He is a very good mentor and a wonderful person to work with. I would also like to thank Drs. Jeffrey O. Dawson (University of Illinois), Mark W. Paschke (Colorado State University), Bob McLean, and Floyd W. Weckerly for being on my dissertation committee. I extend my thanks to Dr. Julie Rieder for providing root nodules and soil samples for my research work. I also appreciate the help of my friend Anita Pokharel in the formatting of the thesis. I am sincerely thankful to my lab mates Allana Welsh, Buddy Gaertner, Ghulam Rasul, Joseph Mendoza and Qiong Sha for their help and support. I am cordially thankful to the Department of Biology, Texas State University-San Marcos, the Graduate College, Dr. Michael Forstner, and the Eben-Ellege and Howard D. Schulze Scholarship Foundations for providing financial support for my PhD.

I am grateful to my parents, brothers and other family members for their love, kind prayers and sincere wishes. My sincere word of thanks goes to Dr. Sajjad Mirza for his valuable guidance and continuous encouragement throughout my studies.

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ABSTRACT

SAPROPHYTIC GROWTH AND FATE OF *FRANKIA* STRAINS IN SOIL

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August 2009

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Frankia are nitrogen fixing bacteria that form root nodules with more than 200 actinorhizal plant species. In nature, *Frankia* are found in soil and in root nodules of specific host plants. Due to their low abundance in soil and difficulties to isolate them, most studies on *Frankia* focus on populations in root nodules, which are natural locale of enrichment for *Frankia*. As a consequence, little is known about the ecology of *Frankia* in soil. The work presented in this PhD. dissertation focused on two basic objectives: 1) to elucidate the effects of specific environmental conditions on the fate of introduced

Frankia strains in soil microcosms, and 2) to highlight the limitations of plant bioassay analyses in describing the diversity of *Frankia* in soil.

Work on the first objective provided evidence that *Frankia* strains differ with respect to their ability to utilize specific organic compounds (chapter II, and III), and that -within the *Alnus* host infection group- the utilization of leaf litter is a specific trait of few *Frankia* strains, and reflected in their taxonomic position (chapter III). Results for the second objective demonstrated that the host plant species has a large effect in the selection of *Frankia* strains from soil for potential nodule formation (chapter IV), and that this effect results in large differences between *Frankia* populations detected directly in soil and those in root nodules (chapter V). Consequently, the choice of the capture plant species has a significant effect in bioassays on diversity estimates of frankiae in soil.

Specific results for the first objective were that *Frankia* strains have the potential to grow saprophytically, with the majority of strains belonging the *Elaeagnus* and *Alnus* host infection groups growing in the rhizosphere of a non-host plant, *Betula pendula*, but not in the surrounding bulk soil. *Casuarina*-infective strains that are generally assigned to the *Alnus* host infection group, however, did not grow in the rhizosphere of *B. pendula*, even though these same strains did grow in the rhizosphere of *Casuarina cunninghamiana*. In contrast to results obtained for the rhizosphere of *B. pendula*, saprophytic growth on leaf litter as a C source was restricted to a small fraction of *Frankia* strains that all belonged to a distinct phylogenetic cluster within the *Alnus* host infection group. These results demonstrated that saprophytic growth of frankiae was a common trait for most members of the genus, and the supporting factors for growth (i.e. carbon utilization capabilities) varied with host infection group and phylogenetic affiliation of the strains. These studies also

provided information on the usefulness of comparative *nifH* gene sequences analyses to distinguish *Frankia* clusters within the *Elaeagnus* and *Alnus* host infection group, with comparable assignments of strains but better resolution than the previously used insertion in the 23S rRNA gene.

Specific results for the second objective highlighted the potential role of host plant species in the selection of nodule-forming frankiae from soil in bioassays with two *Morella*, three *Elaeagnus* and one *Shepherdia* species as capture plants. Diversity of frankiae was larger in nodules on both *Morella* species than in nodules formed on the other plant species, and none of the plants captured the entire diversity of nodule-forming frankiae. The distribution of clusters of *Frankia* populations and their abundance in nodules was unique for each of the plant species with only one cluster being ubiquitous and most abundant while the remaining clusters were only present in nodules of one (six clusters) or two (two clusters) host plant species. These results demonstrated large effects of the host plant species in the selection of *Frankia* strains from soil for potential nodule formation, and thus the significant effect of the choice of capture plant species in bioassays on diversity estimates in soil.

Meta-analysis including sequences previously published for cultures, for uncultured frankiae in root nodules of *Morella pensylvanica* formed in bioassays, and gene clone libraries for the respective soils displayed large differences in cluster assignments between sequences retrieved from clone libraries and those obtained from nodules, with assignments to the same cluster only rarely encountered for individual soils. These results demonstrated large differences between detectable *Frankia* populations in soil and those in root nodules indicating the inadequacy of bioassays for the analysis of frankiae in soil and the role of plants in the selection of frankiae from soil for root nodule formation.

CHAPTER I

GENERAL INTRODUCTION

Nitrogen is an essential plant nutrient, and in most soils it is a limiting factor for plant growth. Although nitrogen comprises nearly 80% of the earth's atmosphere in form of dinitrogen gas (N₂), plants are unable to use this form of nitrogen for their growth and development. Plants fulfill their nitrogen requirements from decomposing organic matter, chemical fertilizer or through biological nitrogen fixation in which certain microorganisms reduce the atmospheric N₂ into ammonium with the help of the enzyme nitrogenase. The ability to reduce N₂ is restricted to members of the Domains Bacteria and Archaea, with a large variety of bacteria belonging to different phylogenetic groups. Nitrogen fixing organisms are found in the α -, β -, γ -, δ -, and ϵ -subclasses of Proteobacteria, whereas nitrogen-fixation in Archaea is less common (Mehta *et al.*, 2003; Mehta & Baross, 2006; van der Maarel *et al.*, 1999). The total amount of nitrogen fixed by these microbes that are either free living, or associated or even symbiotic with plants was estimated to be 100-175 million metric tons per year (Burns & Hardy, 1975).

Nitrogen fixation by symbiotic bacteria is used in agriculture or silviculture to enhance nitrogen availability to specific crop plant species. In agricultural systems the most commonly used symbiotic bacteria are those of or related to the genus *Rhizobium* that form root nodules with leguminous plants, many of which are valuable crop plants

(Zahran, 1999). In this association, the crop plants benefit directly from their nitrogen-fixing bacterial partner. This is different for the most prominent nitrogen-fixing symbiotic association used in silviculture that is represented by bacteria of the genus *Frankia* and actinorhizal plants, even though leguminous associations are used occasionally as well (Nichols *et al.*, 2001; Sprent, 2005; Sprent & Parsons, 2000).

Members of the genus *Frankia* are generally described as nitrogen-fixing actinomycetes that form root nodules in symbiosis with more than 200 species of non-leguminous woody plants in 25 genera of angiosperms (Benson & Silvester, 1993; Huss-Danell, 1997b; Schwintzer & Tjepkema, 1990). The genus *Frankia* is the only genus within the family *Frankiaceae*, which -together with the families *Acidothermaceae*, *Geodermatophilaceae*, *Microsphaeraceae* and *Sporichthyaceae*- is assigned to the suborder *Frankineae*, one out of ten suborders within the order *Actinomycetales* (Stackebrandt *et al.*, 1997).

Frankia forms root nodules (Fig. 1) in symbiosis with host plants such as *Alnus*, *Elaeagnus* and *Casuarina* species which enables them to grow on sites with restricted nitrogen availability (Akkermans *et al.*, 1992). Actinorhizal plants are uniquely successful pioneer plants, frequently establishing themselves after flooding, fires, landslides, glacial activity, as well as volcanic eruptions (Dawson, 1990). They grow on soils with a wide range of properties (Dawson, 1990; Dixon & Wheeler, 1983). They physically enhance the stability of soils with their well-developed root systems (Knowlton & Dawson, 1983) and increase nitrogen mineralization rates in soil thereby enhancing nitrogen availability and improving the quality of impoverished soils.

Unfortunately, only very few actinorhizal plants produce lumber of sufficient economical value, e.g. some *Casuarina* and *Alnus* species (Anonymous, 1980; Dawson, 1986; Wheeler *et al.*, 1986), which means that the benefit of enhanced nitrogen availability for many *Frankia*-actinorhizal plant systems does not translate into a direct benefit for a crop plant.



Figure 1.1: Root nodules. Root nodules of *Alnus glutinosa*. Dime represents size marker.

Many actinorhizal plants are therefore used for reforestation and reclamation of depauperate, nitrogen-limiting soils, but only few species (i.e. a few *Alnus*, *Elaeagnus* and *Casuarina* species) in forestry and agroforestry (Gordon & Dawson, 1979; Gordon, 1983; Roy *et al.*, 2007). Several *Alnus* species, for example, are used as a source of timber themselves or for the production of fuel wood, but also as nurse trees in mixed

plantations with more valuable tree species, i.e. by interplanting them with suitable tree crops such as walnut (Dawson, 1983; Dawson, 1986; Gordon & Dawson, 1979; Gordon, 1983). Mixed plantations of *Alnus* or *Elaeagnus* spp. and valuable tree species are a proven silvicultural practice that takes advantage of the ability of actinorhizal plants to increase soil nitrogen availability to associated tree crops. The total nitrogen input through nitrogen fixation of the symbiosis between actinorhizal plants and *Frankia* can range from 60 to 320 kg N ha⁻¹ y⁻¹ (Paschke *et al.*, 1989). However, since mainly nitrogen from mineralized organic matter is utilized by the associated tree crops, rates of nitrogen mineralization more accurately reflect the influence of actinorhizal plants on soil fertilization than estimates of total nitrogen input (Dawson, 1990). The amount of nitrogen from mineralization of organic matter may range between 48 and 185 kg N ha⁻¹ y⁻¹ for alder stands (Paschke *et al.*, 1989) and can even reach amounts of 236 kg N ha⁻¹ y⁻¹ for plantations with autumn-olive (*Elaeagnus umbellata* Thunb.) (Paschke *et al.*, 1989).

Successful establishment of mixed plantations comprised of actinorhizal and other plants depends on many site factors, but also on the species interplanted which, for example, should not compete unduly with one another for light or nutrients. Several hardwood species have shown improved growth when interplanted with black alder (*Alnus glutinosa* (L.) Gaertn.) or with autumn-olive (*E. umbellata*) (Funk *et al.*, 1979; Schlesinger & Williams, 1984). These species include black walnut (*Juglans nigra* L.), a highly prized timber species of the temperate deciduous forests of the central United States (Paschke *et al.*, 1989). It is known that the efficiency of the symbiosis between *Frankia* and woody plants of the genus *Alnus* is largely determined by environmental factors such as the soil pH (Crannell *et al.*, 1994; Griffiths & McCormick, 1984), the soil

matric potential (Dawson *et al.*, 1989; Schwintzer, 1985), and the availability of elements such as nitrogen (Kohls & Baker, 1989; Thomas & Berry, 1989) or phosphorus (Sanginga *et al.*, 1989; Yang, 1995). Genotypes of both partners of this symbiosis ultimately determine the success of the symbiosis under a given set of environmental conditions (Hall *et al.*, 1979; Prat, 1989). An improvement of the symbiosis for economic purposes therefore requires the selection of optimal growth sites, but also an optimal combination of plants of interest, e.g. forest ecotypes of *Alnus glutinosa*, and superior genotypes of *Frankia* as inoculum (Hall *et al.*, 1979; Hilger *et al.*, 1991; Wheeler *et al.*, 1991).

While properties of sites and plants are relatively easy to assess, quantitative analysis of specific *Frankia* populations in soil and their interaction with plants and sites, however, is methodologically extremely challenging. It is therefore not surprising that information on the fate of specific *Frankia* populations in soil, and the resulting consequences on the interaction with its host plant (i.e. its nodulation) is scarce. While many studies have shown positive effects of inoculation on plant establishment and subsequent growth performance (Hilger *et al.*, 1991; Houwers & Akkermans, 1981; Wheeler *et al.*, 1986), little is known about the establishment of inoculated strains in root nodules and in soils. Only a few studies have demonstrated that inoculated strains successfully competed with indigenous populations (Nickel *et al.*, 1999; Nickel *et al.*, 2001) or with other inoculated strains for nodule formation (Martin *et al.*, 2003), and that they can persist and remain infective in soils for some time (McEwan *et al.*, 1999; Smolander *et al.*, 1987).

This lack of information is hampering efficient inoculation programs with *Frankia* strains on alders that require suitable inoculum strains with high nitrogen-fixing capacity and compatibility in combination with the ability to form nodules promptly, to persist in the particular soil (e.g., in the presence of *J. nigra*) and to compete with indigenous *Frankia* strains. As a consequence, large variations in yield improvement have discouraged many foresters from making mixed plantations between actinorhizal plants and valuable crop tree species, although many studies in silviculture have shown the usefulness of such plantings in the past (Gordon & Dawson, 1979; Gordon, 1983). The difficulties of establishing a manageable system between crop trees such as, for example, *J. nigra* and the often used nurse tree *A. glutinosa* have drawn into question the usefulness of the symbiosis between *A. glutinosa* and the nitrogen-fixing actinomycete *Frankia* as a system to increase the content and the availability of nitrogen in managed forests (Neave & Dawson, 1989; Rietfeld *et al.*, 1983). A first step towards understanding the complex interactions between actinorhizal plants and *Frankia* strains, and one with potential practical benefits in reforestation and agroforestry, is to investigate interactions in systems with a limited range of variability in plant-*Frankia* composition (i.e. combinations of specific host plants and *Frankia* strains as inoculum) and standardized soil environmental conditions (i.e. soil mesocosms that contain a natural microbial community, but exclude as many variables as possible from the potentially highly complex system under study). In these systems, manipulations of environmental conditions could retrieve information on potentially selective conditions for specific *Frankia* strains that might be useful to either shift populations in the indigenous *Frankia* community towards desirable strains, or help inoculated strains to persist long-term.

Quantitative analysis of specific *Frankia* populations in soil are methodologically extremely challenging since studies of the ecology of natural or introduced populations of *Frankia* are limited by problems encountered with its isolation and identification (Benson & Silvester, 1993; Schwintzer & Tjepkema, 1990). In the presence of other bacteria it is virtually impossible to obtain *Frankia* in pure culture, and only one successful attempt to isolate *Frankia* from soil has been reported (Baker & O'Keefe, 1984). *Frankia* is usually isolated from root nodules, a natural locale of enrichment for this organism. Different isolation procedures have successfully been used during the last 30 years and many isolates are available (Schwintzer & Tjepkema, 1990). However, even for *Frankia* strains in root nodules, no general isolation protocols have been developed and consequently only a small percentage of isolation attempts succeed. Despite the inability of quantitative isolation, the ability to obtain isolates from nodules has resulted in a considerable amount of information on *Frankia* strains isolated from root nodules and on their interaction with their host plants (see Benson & Silvester, (1993); Huss-Danell, (1997) for review).

Studies of *Frankia* populations in soil have until recently been based solely on plant bioassays in which a quantification of the nodulation capacity on a specific host plant (expressed as nodulation units g^{-1} soil) is used to describe the infective *Frankia* population. This approach includes regression and most probable number (MPN) methods in which host plants inoculated with serial dilutions of *Frankia*-containing samples are statistically analyzed on the basis of nodule formation (Huss-Danell & Myrold, 1994). Using these methods, nodulation units between 0 and 4600 g^{-1} soil have been obtained for different soils (Myrold *et al.*, 1994a). The nodulation capacity of soil from birch-stands, for example, appeared to be as high or higher as that of soil from

alder-stands (Smolander, 1990a). Since birch is a non-host plant for *Frankia*, these results and those in several additional studies (e.g. (Smolander & Sundman, 1987; Smolander *et al.*, 1990a; Smolander & Sarsa, 1990b) demonstrated that members of the genus *Frankia* can survive and remain infective in soils that are devoid of host plants (Maunuksela *et al.*, 1999; Smolander, 1990c).

In recent years nodulation-dependent detection methods have increasingly been supplemented or replaced with molecular methods, which are powerful tools to analyze *Frankia* populations directly in their habitat (Akkermans *et al.*, 1991; Hahn *et al.*, 1999; Mullin & Dobritsa, 1996). Molecular methods target sequences of macromolecules (DNA, mRNA or rRNA), that characterize organisms and their potential metabolic activities, rather than the organisms themselves. These methods therefore allow researchers to study microbial communities and specific populations unaffected by the limitations of culturability and to get an indication of their *in situ* abundance and activity (see Amann *et al.*, (1995) for review).

Initial attempts to quantify *Frankia* populations directly in soil used rRNA as the target molecule. In RNA extracts from 1 g of soil, genus-specific hybridization with oligonucleotide probes resulted in the detection of frankiae with an estimated detection limit of 10^4 cells per g (Hahn *et al.*, 1990). This detection limit was comparable with that obtained by PCR with 10^4 genomic units [g soil dry wt.]⁻¹ (Myrold & Huss-Danell, 1994a). A genomic unit was defined as the amount of frankiae containing a single gene, though frankiae appeared to contain two rRNA operons. The detection limit could be increased to 10 genomic units [g soil dry wt.]⁻¹ by optimizing PCR conditions and using

booster PCR (Myrold & Huss-Danell, 1994a). Today, quantification of *Frankia* populations usually employs PCR-MPN using nested (Myrold & Huss-Danell, 1994a) or booster PCR (Picard *et al.*, 1992). Comparison of this method of quantification with that of plant bioassays revealed that the fraction of *Frankia* capable of nodulation, which ranged between 0.2 and 2,940 nodulation units [g soil dry wt.]⁻¹, was just a small portion of the total population of *Frankia* as measured by genomic units, which ranged from 2,000 to 92,000 genomic units [g soil dry wt.]⁻¹ (Myrold & Huss-Danell, 1994a). These results suggested that the nodulation capacity of a soil was controlled largely by the physiological and host-compatibility status of the *Frankia* populations rather than by the total population size (Myrold & Huss-Danell, 1994a).

This assumption is supported by studies in which only one population of *Frankia* was detected in nodules of the host plant at the respective site by *in situ* hybridization, though different *Frankia* populations were detected in soil by PCR (Zepp *et al.*, 1997b). Comparable results were obtained in a follow-up study with an approach combining plant bioassay and PCR-assisted detection of *Frankia* populations directly in soil when *Frankia* populations in three soils devoid of actinorhizal plants were studied (Maunuksela *et al.*, 1999). Depending on whether the soil originated from a birch-, pine- or spruce-stand, different *Frankia* populations were found in the nodules of the capture plants. However, no differences in the diversity of the total *Frankia* populations were obtained by nested PCR on nucleic acids extracted from the respective soils (Maunuksela *et al.*, 1999). Although the PCR-based results indicated a similar diversity of the total *Frankia* population in soils of the birch-, pine- and spruce-stands, PCR products do not necessarily reflect the abundance of the target sequences in the original sample (Suzuki &

Giovannoni, 1996). Therefore, the size of the *Frankia* populations detected may differ significantly in soils of the birch-, pine- and spruce-stands.

Growth of frankiae in soil is affected by carbon resource availability. It has been shown that growth of *Frankia* is enhanced in the rhizosphere of both host and non-host plants under axenic conditions, most obviously influenced by root exudates that are easily available carbon-resources (Rönkkö *et al.*, 1993), and that organic material in soil (i.e. leaf litter representing slowly available carbon resources) affects the nodulation capacity of two *Frankia* strains (ArI3, and Ag45/Mut15 representing two subgroups within the *Alnus* host infection group) in the absence of plants (Nickel *et al.*, 2001). Leaf-litter-amendment to non-vegetated soil resulted in the establishment of *Frankia* populations with high specific N₂-fixing capacities inoculated into this soil, or a shift in the composition of the indigenous *Frankia* populations to that one represented by the established inoculated strain (Nickel *et al.*, 2001). These populations remained infective on their host plants by successfully competing for nodule formation with other indigenous or inoculated *Frankia* populations and thereby increased plant growth performance (Nickel *et al.*, 2001).

So far, bioassays and PCR-based analysis methods indicated that *Frankia* populations in root nodules represent only a small portion of all *Frankia* populations present in a soil, and that nodulation by specific populations was affected by vegetation and environmental factors (Hahn *et al.*, 1999). Based on these results shifts in competitive ability of specific *Frankia* populations with respect to root nodule formation were suggested to be triggered by environmental determinants that favor saprophytic (i.e. free-living) growth of one

population in soil over that of other populations (Maunuksela *et al.*, 1999; Nickel *et al.*, 2001). This speculation was based on the assumption that only actively growing frankiae could nodulate and that larger populations would have a competitive advantage over smaller populations. These assumptions, however, did not consider potential drawbacks of both bioassays and PCR-based analysis methods that render quantitative analyses data highly unreliable and speculative.

Apart from the need for large numbers of test plants, the use of bioassays is hampered by its selectivity, since only nodule-forming *Frankia* populations can be detected. Since a nodule can theoretically be induced by a single spore, a hyphal fragment or a colony, the correlation of the nodulation unit with cell numbers remains problematic (Myrold *et al.*, 1994a). Adding to this problem is the uncertainty whether the infectious *Frankia* particle in soil is really an actively growing organism present in vegetative form (i.e. in long filaments as in pure culture, in short fragments or in single cell form) or a spore activated by exudates of the capture plant used in the bioassay. In the latter case, nodule formation would reflect properties of the capture plant rather than of the soil to be analyzed.

Furthermore, questions on *Frankia* populations belonging to host infection groups other than the test plants (Baker, 1987), or on non-nodulating *Frankia* populations of the same host infection group (Hahn *et al.*, 1988) are neglected. Other drawbacks of the bioassay include the failure to analyze non-competitive *Frankia* populations, including those present in low numbers, the inability to quantify competition for infection on the capture plant between different *Frankia* populations in a sample, and variable compatibilities of host plants to *Frankia* populations (Huss-Danell & Myrold, 1994). For example, the number of nodulation units g^{-1} soil varied from 10 to 380 depending on the cultivation

conditions as well as on the capture plant used, with nodule numbers consistently being greatest on *Alnus rubra*, less on *A. incana*, and least on *A. glutinosa* (Huss-Danell & Myrold, 1994). Variable compatibilities of capture plants were recently confirmed, though with contradicting results to (Huss-Danell & Myrold, 1994) with nodule numbers being higher on *A. glutinosa* than on *A. incana* (Maunuksela *et al.*, 2000). Variable compatibilities of capture plants in bioassays could also potentially impact nodulation by specific *Frankia* strains as indicated previously (Maunuksela *et al.*, 2000). Nodulation capacities could therefore reflect the effect of the plant species on a specific *Frankia* population with respect to nodulation rather than represent a quantitative picture of the overall structure of nodule-forming populations.

Artifacts might not only be introduced by bioassays, but also by molecular analysis tools. Although the most prominent advancement in studies on the ecology of *Frankia* has been credited to the use of the PCR technique, PCR products do not necessarily reflect the abundance of the target sequences in the original sample (Suzuki & Giovannoni, 1996), and are usually not differentiating between vegetative and dormant stages (although real-time PCR methods have been developed that have the potential to address this problem by using genes on DNA and the respective mRNA) (Okano *et al.*, 2004; Skovhus *et al.*, 2004; Stults *et al.*, 2001; Tajima *et al.*, 2001). Thus, although bioassays and PCR-based analysis methods have been used as quantitative analyses tools by many researchers in the past, results obtained so far might seriously be biased or even represent artifacts of the analysis protocol.

Bioassay- and PCR-based detection of frankiae is currently complemented by hybridization assays using specific oligonucleotides and ribosomal RNAs (rRNAs) in as probe/target system. The most promising results were retrieved by the *in situ* hybridization technique that focuses on the microscopic detection of labeled probes hybridized to target sequences in fixed cells of frankiae at different taxonomic levels (Hahn *et al.*, 1997; Zepp *et al.*, 1997a; Zepp *et al.*, 1997b). This method avoids the *in vitro* amplification of the target sequences because RNA molecules naturally abundant in cells are used as targets. In addition to quantitative information on the presence and abundance of the target organism, information on their morphology (i.e. the occurrence in filaments or cocci, as spores or vesicles, single cells, small fragments or colonies) can be retrieved, which can be related -to a certain extent- to a potential physiological status (i.e. as dormant or vegetative cells, or potentially carrying out a metabolic function such as e.g., nitrogen-fixation if hybridized with probes targeting mRNA of *nifH*, etc.). Information on the abundance of the target organism can be used to determine their biomass after determination of biovolumes by image analysis (Schönholzer *et al.*, 1999; Schönholzer *et al.*, 2002), and on their localization (e.g. in nodules, soil, and potential interactions, e.g. on the root surface).

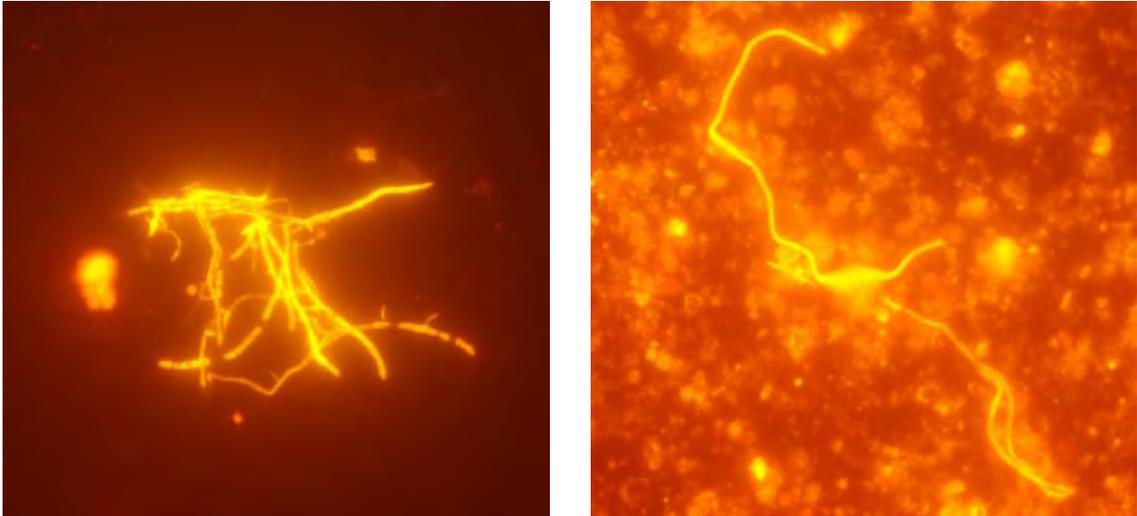


Figure 1.2: *Frankia* strain Ag45/Mut15 in soil and liquid medium. Growth of *Frankia* strain Ag45/Mut15 grown on ground leaves of *Alnus glutinosa* as sole C- and N-source for two months (left side) and in soil amended with the 5% leaf litter of *Alnus glutinosa* (right side) after two months of incubation. *In situ* hybridization with fluorescently labeled probes was used to detect the *Frankia* cells in to two different environments. Since these probes targeted the rRNA molecules actively growing filaments can be seen. It also shows the differences in the form of growth i.e. colonies in liquid culture (left) and individual filament in soil (right).

In situ hybridization for the first time allows the differentiation and exact quantification of *Frankia* strains belonging to different subgroups. *Frankia* strains belonging to the same subgroup, however, cannot be differentiated with the currently available probe/target systems, i.e., oligonucleotides and rRNAs. This probe/target system for frankiae is based on sequences of a large insertion in Domain III of the 23S rRNA specific for gram-positive bacteria with a high DNA G+C content (Roller *et al.*, 1992). Remarkable variation in this region was observed within the genus *Frankia* when sequences of *Frankia* strains belonging to the *Alnus* host infection group (Hönerlage *et*

al., 1994; Maunuksela *et al.*, 1999) and those of *Frankia* strains belonging to other host infection groups were compared (Hönerlage *et al.*, 1994). The results confirmed the classification of the strains into host infection groups (Hönerlage *et al.*, 1994) as proposed by 16S rRNA sequence analysis (Normand *et al.*, 1996). In addition, strains belonging to the *Alnus* host infection group could be separated roughly into four subgroups, three containing typical nitrogen-fixing strains and a fourth exclusively of non-nitrogen-fixing strains (Hönerlage *et al.*, 1994).

Despite the limitation to discriminate between closely related strains, *in situ* hybridization with rRNA-targeted (and potentially mRNA-targeted) oligonucleotide probes is the only method available to advance the field by simultaneously examining three crucial parameters in soil or soil mesocosms: 1. the quantification of particular strains or subgroups, 2. the differentiation of their presence as inactive spores, active filaments, or differentiated into vesicles, hyphae and/or spores in root nodules, and 3. the *in situ* localization of the various subgroups and cell types in the soil relative to the tree roots. Since changes in *Frankia* soil populations could have profound effects on the establishment, survival, and ecological function of widespread terrestrial plant communities with actinorhizal components, environmental factors affecting the fate of specific *Frankia* populations in soil and their competitive ability with respect to nodule formation need to be assessed, and their effects quantified. *In situ* hybridization allows for ecophysiological studies of *Frankia* populations in soil and opens the door to more sophisticated studies of environmental factors such as the quality of organic material, soil pH, soil matric potential, or the availability of elements such as nitrogen or phosphorus on the dynamics of indigenous or introduced *Frankia* populations in plants and soil.

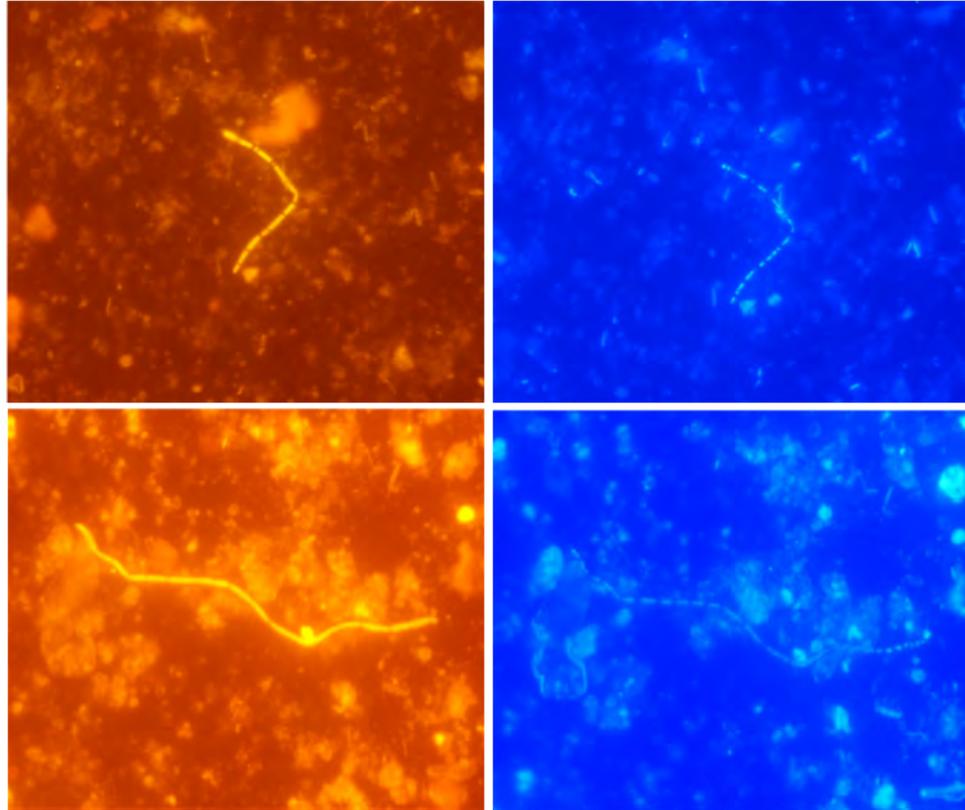


Figure 1.3: *Frankia* strain Ag45/Mut15 in soil at the time of inoculation and after one month of incubation. Actively growing cells of the *Frankia* strain Ag45/Mut15 in soil at the time of inoculation (upper panel) and after one month of incubation in soil amended with organic matter (lower panel). The cells were hybridized with fluorescently labeled *Frankia* specific probe (left side) and DNA was stained with DAPI (right side).

Objective: Two basic objectives were addressed in this Ph.D. study. The first objective was to elucidate the effects of specific environmental conditions on the fate of introduced *Frankia* strains in soil microcosms. Since frankiae are heterotrophic organisms, the most prominent environmental factor affecting growth of frankiae in soil is carbon (C) availability in the form of organic material. Previous studies using axenic conditions have shown that growth of *Frankia* is enhanced in the rhizosphere of host and non-host plants,

most obviously influenced by root exudates that are easily available C sources (Rönkkö *et al.*, 1993). Additionally, less labile C sources like leaf litter affected the nodulation capacity of two *Frankia* strains (ArI3, and Ag45/Mut15 representing two subgroups within the *Alnus* host infection group) in the absence of plants (Nickel *et al.*, 2001). The effect of these two C sources, i.e. plant root exudates and leaf litter, on the fate of different *Frankia* populations in soil was assessed in two studies (chapter II, and III) to address two hypotheses:

- 1) *Frankia* strains differ with respect to their ability to utilize specific organic compounds, and
- 2) Within the *Alnus* host infection group, the utilization of leaf litter is a specific trait of few *Frankia* strains, and reflected in their taxonomic position.

The second objective was to highlight the limitations of plant bioassay analyses in describing the diversity of *Frankia* in soil. Plant bioassay analyses have been the basis for many studies on the diversity and geographic distribution of *Frankia* in soil (Clawson *et al.*, 1997; Clawson *et al.*, 2004; Gtari *et al.*, 2004; Gtari *et al.*, 2007; Jeong *et al.*, 1999; Mirza *et al.*, 1994; Ritchie & Myrold, 1999) despite many problems concerned with the selectivity of the host plant, i.e. the detection of only nodule-forming populations (Baker, 1987), or the failure to analyze the competitive ability of *Frankia* and to consider variable compatibilities of host plants for specific genotypes of *Frankia* (Huss-Danell & Myrold, 1994; Maunuksela *et al.*, 2000). Two hypotheses were addressed in two studies (chapter IV, and V):

- 1) The host plant species has a large effect in the selection of *Frankia* strains from soil for potential nodule formation, and thus the choice of the capture plant species has a significant effect in bioassays on diversity estimates of frankiae in soil
- 2) This effect will result in large differences between *Frankia* populations detected directly in soil and those in root nodules.

A major outcome of the proposed study will be the determination of differences in C source utilization of different *Frankia* strains that have the potential to serve as inoculum. Amendments with specific C sources could either result in population shifts within indigenous *Frankia* populations, or help establish those that have been introduced. The information retrieved will be critical for the establishment of an effective symbiosis from the applied point of view, e.g. by selecting effective strains and providing suitable environmental conditions that might result in long term establishment of the microsymbiont and growth increment in valuable tree crops. The study will also demonstrate the role of plants in the selection of frankiae from soil for root nodule formation and the inadequacy of bioassays for the analysis of frankiae in soil, and highlight the need for methods enabling the direct analyses of *Frankia* populations in terrestrial environments. These methods will be necessary to expand investigations on saprophytic growth of frankiae in soil and to analyze potential effects of the growth state and occurrence of frankiae in soil on root nodule forming capacity.

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CHAPTER II

SAPROPHYTIC GROWTH OF INOCULATED *FRANKIA* SP. IN SOIL MICROCOSMS

Abstract

In situ and dot blot hybridization were used as tools to quantify growth of two *Frankia* strains inoculated alone or together into non-sterile soil microcosms with ground leaf litter of *Alnus glutinosa* as sole carbon (C) and nitrogen (N) sources. A significant increase in cell numbers and filament length was observed during the first 6 weeks after inoculation for strain Ag45/Mut15, both alone and in mixed culture with strain ArI3, followed by a decrease until the end of the study after 12 weeks. The number of filaments remained unchanged. In contrast, cell numbers and filament length of strain ArI3 were reduced significantly during the first 2 weeks and undetectable for the remainder of the study. These results were comparable to those obtained in sterile mineral medium amended with leaf litter of *A. glutinosa*, although reductions in cell numbers and filament length were less pronounced than in soil microcosms. In concomitant control studies without leaf litter amendments for both experimental setups, filaments of both strains could only be detected immediately after inoculation. These results were matched in all experimental setups by concomitant shifts in rRNA content of both strains, i.e.,

an immediate decline in rRNA content for strain ArI3 after inoculation, and an increase in rRNA content followed by a late decline during incubation for strain Ag45/Mut15. These results demonstrated that *Frankia* strain Ag45/Mut15 could grow saprophytically in soil with complex C and N sources such as leaf litter, while growth of strain ArI3 was not supported.

Keywords: *Frankia*, hybridization, image analysis, rRNA, saprophytic growth

Introduction

Actinorhizal plants are characterized by their ability to form root nodules in symbiosis with the nitrogen-fixing actinomycete *Frankia* which enables them to grow on sites with restricted nitrogen availability (Akkermans *et al.*, 1992). They are uniquely successful pioneer plants, frequently establishing themselves after flooding, fires, or landslides (Dawson, 1990). In forestry and agroforestry, actinorhizal plants such as *Alnus* sp. or *Elaeagnus* sp. are therefore used for reforestation and reclamation of depauperate, nitrogen-limiting soils, but also in mixed plantations with valuable tree species taking advantage of the ability of the actinorhizal plants to increase soil nitrogen availability to associated tree crops (Gordon & Dawson, 1979; Gordon, 1983).

The efficiency of the symbiosis between actinorhizal plants and *Frankia* is affected by environmental factors (Crannell *et al.*, 1994; Dawson *et al.*, 1989; Kohls & Baker, 1989; Thomas & Berry, 1989; Yang, 1995), but ultimately determined by the genotypes of both partners (Hall *et al.*, 1979; Prat, 1989). An improvement of the symbiosis for economic purposes therefore requires the selection of not only optimal growth sites for hosts and endosymbionts, but also an optimal combination of plants of interest and genotypes of *Frankia* present in a soil or introduced as inoculum (Hall *et al.*, 1979; Hilger *et al.*, 1991;

Wheeler *et al.*, 1991). Inoculation, for example, has been shown to increase plant growth performance and nitrogen availability (Nickel *et al.*, 1999; Steele *et al.*, 1989; Strukova *et al.*, 1996), and has allowed the establishment of nitrogen-fixing *Frankia* populations in root nodules under conditions that did not favor vesicle formation in nodules formed by the indigenous *Frankia* population (Nickel *et al.*, 1999). For long-term effects, however, the introduced strains should not only compete with the indigenous *Frankia* populations for nodule formation, but should also remain active in the nodules and survive in soil.

Plant bioassays in which a quantification of the nodulation capacity on a specific host plant is used to describe the infective *Frankia* population (Huss-Danell & Myrold, 1994) have demonstrated that members of the genus *Frankia* can survive and remain infective in soils that are devoid of host plants (Arveby & Huss-Danell, 1988; Gtari *et al.*, 2004; Maunuksela *et al.*, 1999; Paschke & Dawson, 1992; Smolander & Sundman, 1987; Smolander *et al.*, 1988). For soils from stands with *Betula pendula*, a non-host plant for frankiae, the nodulation capacity appeared to be as high or even higher than that of soils from stands with *Alnus incana*, a host plant for frankiae (Smolander, 1990a). These results suggest that frankiae not only persist in soils devoid of host plants, but proliferate and thus grow saprophytically.

Support for potential saprophytic growth of frankiae in soils is provided by previous studies that have shown growth of *Frankia* strains in the rhizosphere of host and non-host plants under axenic conditions, most obviously influenced by root exudates that are easily available carbon (C) resources (Rönkkö *et al.*, 1993; Smolander, 1990a). In the absence of plants, amendment of soils with slowly available C resources such as leaf litter affected the nodulation capacity of different *Frankia* strains (Nickel *et al.*, 2001). Other

studies found that leaves of *Casuarina* sp. promoted growth of *Frankia* strains infective on *Casuarina* (Zimpfer *et al.*, 1999). Although these observations as well as the fact that *Frankia* can be grown in pure culture indicate that *Frankia* has at least the potential to grow saprophytically in soil, no direct evidence for saprophytic growth in soil is available (Huss-Danell, 1997).

The aim of this study was therefore to provide direct evidence for saprophytic growth of *Frankia* strains in soil. These studies were based on the monitoring of basic growth characteristics (i.e., cell numbers and filament length) in time of two strains inoculated into non-sterile, C-limited soil that was amended with leaf litter. *In situ* hybridization with fluorescent probes allowed us to specifically analyze images of each strain and to distinguish them from the indigenous microbial community. These studies were accompanied by analyses of cell numbers and filament length of these strains in basal mineral medium amended with leaf litter. In contrast to soil microcosms, amended mineral medium was sterilized to investigate potential inhibitory effects of inoculation on growth, i.e., biotic factors leading to soil microbiostasis (Ho & Ko, 1985). The results obtained by image analyses were subsequently verified by dot blot hybridization quantifying contents of 23S rRNA for these strains in time.

Materials and Methods

Experimental setup: Microcosms were established for 4 treatments in 2-ml cryotubes that were sampled destructively at 6 time steps (i.e., after 0, 2, 4, 6, 8, and 12 weeks of incubation). For each treatment and time, 5 tubes were harvested with three tubes used for RNA extraction, and 2 tubes for cell fixation. The first two treatments contained 500 mg of soil (dry wt.) obtained from a natural stand of *A. glutinosa* (Ettiswil, Switzerland)

(Zepp *et al.*, 1997a). This soil was characterized by a low content of organic material (0.02%) (Nickel *et al.*, 2001) and the presence of different *Frankia* populations of the *Alnus* host infection group, i.e., subgroups IIIa, IIIb and IV (Zepp *et al.*, 1997b) that had previously been established based on sequence similarities of an actinomycetes-specific insertion in Domain III of the 23S rRNA (Hönerlage *et al.*, 1994). One treatment contained the original soil only, while the second treatment contained the soil amended with 5% (wt/wt) leaf litter of *A. glutinosa* ground to a particle size of about 0.1 mm. Both soil microcosm treatments were not sterilized, while the remaining medium treatments were sterilized. The first of these medium treatments consisted of basal mineral DPM medium (Meesters *et al.*, 1985) without carbon (C) and nitrogen (N) sources, and the second of the same medium amended with 1% (wt/vol) ground leaf litter of *A. glutinosa*.

For each treatment, 4 inoculation schemes with *Frankia* strains were established (no inoculation, inoculation with strains Ag45/Mut15 (Hahn *et al.*, 1988) or ArI3 (Murry *et al.*, 1984) alone, or inoculation with both strains in combination). These strains that represented *Alnus* host infection groups IIIa and IV, respectively (Hönerlage *et al.*, 1994) were grown for two weeks in DPM medium containing propionate and NH₄Cl as C and N source, respectively (Meesters *et al.*, 1985). Cells were harvested by centrifugation, washed in distilled water twice, and homogenized in basal DMP mineral medium without C and N sources using a tissue homogenizer. These cells were diluted to comparable densities in basal DPM mineral medium, or in this medium containing 1% leaf litter. A final volume of 500 µl of homogenate containing either the individual strains or a mixture of both strains at the same density as in homogenates for the individual strains

was inoculated into each cryotube. The same volume of medium without *Frankia* strains were used as controls. All samples were mixed and incubated at 30°C in the dark.

***In situ* hybridization and image analyses:** For *in situ* hybridization, samples (n=2 cryotubes per treatment and inoculation scheme, i.e., 32 tubes per time step) were harvested after 0, 2, 4, 6, 8, and 12 weeks and cells fixed in 4% paraformaldehyde in phosphate buffered saline (PBS; 0.13 M NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, pH 7.2) at 4°C for 16 hours (Hahn *et al.*, 1992). Afterwards, samples were washed in PBS and stored in a final volume of 1 ml of 50% ethanol in PBS at -20°C until further use (Amann *et al.*, 1990). Sub-samples of 10 µl were diluted in 990 µl of 0.1 % sodium pyrophosphate, and 10 µl of this dilution spotted on gelatin coated slides (0.1% gelatin, 0.01% KCr(SO₄)₂). Samples were dried at 42°C for 15 minutes, and subsequently dehydrated in 50, 80 and finally 96% ethanol for three minutes each. Cells were then treated with 0.1% lysozyme (Fluka, Buchs, Switzerland, 1 mg corresponding to 37,320U dissolved in 1 ml of 100 mM Tris/HCl, pH 7.5, 5 mM EDTA) for 60 minutes followed by achromopeptidase (60U ml⁻¹) for 20 minutes (Sekar *et al.*, 2003). Slides were rinsed with distilled water and dehydrated in 50, 80, and 96% ethanol for three minute in each.

Hybridizations with Cy-3 labeled probes 23ArI3 (5' CCA GAC ACA TCT CCG AA) or 23Mut(II) (5' CCA CAC ACA CCC CCT AA) (Zepp *et al.*, 1997b) targeting subgroup-specific sequences on an actinomycetes-specific insertion in Domain III of the 23S rRNA were performed in 9 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, 5 mM EDTA, 0.01% SDS, pH 7.2) containing of 30% formamide, 1 µl of probe (25 ng µl⁻¹) and 1 µl of DAPI solution (final conc. 200 ng µl⁻¹) at 42°C for 2 hours. After hybridization, slides were washed with hybridization buffer at room temperature for 15 minutes, rinsed

with distilled water and air-dried. Slides were mounted with Citifluor AF1 solution (Citifluor Ltd., London, UK) and examined with a Nikon Eclipse 80 I microscope, fitted for epifluorescence microscopy with a high-pressure metal halide lamp and filter sets F31 (AHF Analysentechnik, Tübingen, Germany; D360/40, 400DCLP, D460/50, for DAPI detection) and F41 (AHF Analysentechnik; HQ535/50, Q565LP, HQ610/75, for Cy3 detection), respectively. DAPI and Cy3 pictures were taken from the same image using a Nikon DXM 1200F digital camera, and 25 images per treatment and time were analyzed for cell numbers and filament length using Image-Pro[®] Plus (Version 5.1).

Dot blot hybridization: Samples (n=3 cryotubes per treatment and inoculation scheme, i.e., 48 per time step) were harvested after 0, 2, 4, 6, 8, and 12 weeks. Samples without soil were centrifuged, and cell pellets as well as soil samples frozen at -80°C until further use. Pure cultures of *Frankia* strains Ag45/Mut15 and ArI3 as well as *E. coli* DH5 α were used as standards and as positive or negative controls, respectively. Nucleic acid extraction from all samples was based on a bead beating method (Hönerlage *et al.*, 1995) in which 1 g or 0.5 g of zirconium beads (0.1-0.11 mm) were added to cell pellets or to soil samples, respectively, and cryotubes were filled up to 1.5 ml total volume with extraction buffer (200 mM Tris/HCl, pH 8.0; 1.5% LiDS; 300 mM LiCl; 10 mM Na₂EDTA; 1% sodium deoxycholat; 1% Triton X-100; 0.5 mM thiourea; 10 mM dithiothreitol) (Hönerlage *et al.*, 1995).

Cells were disrupted by agitation in a Mini-Bead-Beater-8 (BioSpec Products, Inc, Bartlesville, OK) for 2 minutes at maximum setting. Beads, soil and cell debris were separated from buffer by centrifugation at 14,000 rpm for 1 minute, and nucleic acids released into the supernatant purified by sequential phenol, phenol/chloroform and

chloroform extraction (Sambrook *et al.*, 1989). Nucleic acids were precipitated by centrifugation at 13,000 rpm for 10 minutes after mixing with 1 volume of isopropanol, washed with 70% ethanol, air-dried and finally re-suspended in 30 μ l of sterile distilled water.

For dot blot hybridization, 15 or 25 μ l of re-suspended nucleic acids from liquid or soil samples, respectively, were applied to a nylon membrane (MagnaGraph) with a dot blot manifold (BioRad, Hercules, CA) and fixed by exposure to UV light for 5 minutes. After pre-hybridization for 20 minutes at 38°C in hybridization buffer (0.36 M Na₂HPO₄, pH 7.2, 5% SDS, 1mM Na₂EDTA, 1% bovine serum albumin) (Church & Gilbert, 1984) that included 45% formamide, 100 ng of either digoxigenin-labeled probe 23ArI3 or probe 23Mut(II) were added and hybridized for 16 hours (Hahn *et al.*, 1992). The formation of stable hybrids was shown by binding of an antibody-alkaline phosphatase conjugate (Roche, Indianapolis, IN) to the digoxigenin reporter molecule. Alkaline phosphatase activity was visualized by light emission using CSPD[®] as substrate for chemiluminescence (Roche) and exposure to Kodak X-OMAT AR film according to the manufacturer's instructions. Signal intensities were determined by image analysis of scanned films (Epson 3590 photo scanner) using Image-Pro[®] Plus (Version 5.1) (Media Cybernetics, Inc. Bethesda, MD).

For quantification and controls, rRNA of *Frankia* strains Ag45/Mut 15 and ArI3 and of *E. coli* DH5 α was quantified using a BioMate 3 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Serial dilutions of these rRNAs in 20 μ l DEPC (0.1%) treated water (i.e., 1, 5, 10, 15, and 20 ng for frankiae and 20 ng for *E. coli*) were bound to the membrane and hybridized concomitantly with dilutions of the samples. Regression

analysis with the calibration curve was used to quantify the rRNA content in the samples. Values are presented as mean \pm standard error (SE) (n=3).

Statistical analyses : Values for number of cells and fragment length of frankiae were analyzed by single factor MANOVA and differences among means were detected by using Tukeys HSD test utilizing the statistical software R, version 2.5.0 (www.R-project.org). Values for rRNA content were analyzed by single factor ANOVA and differences among means were detected with Tukeys HSD test. The level of significance for each test was $P < 0.05$.

Results

Epifluorescence microscopy after *in situ* hybridization with specific probes and concomitant staining with DAPI allowed us to visualize both *Frankia* strains Ag45/Mut 15 and ArI3 in soil microcosm and in mineral medium. In treatments without C-resources, i.e., in soil microcosms and in mineral medium without leaf litter amendment, cells could only be seen directly after inoculation (t=0), but not at any other sampling time (data not shown). Inoculation with *Frankia* strains was necessary, since no *Frankia* cells could be detected in non-inoculated soil microcosms at all sampling times. These results corroborated dot blot hybridization results where rRNA of both *Frankia* strains could only be detected directly after inoculation into non-amended soil microcosms and mineral media. Without inoculation, rRNA could not be detected with probes targeting both *Frankia* strains.

In contrast to results on non-amended and non-inoculated soil microcosms and mineral medium, leaf-litter amendment and inoculation resulted in the detection of frankiae in time both by *in situ* and dot blot hybridization. DAPI-stained cells could easily be

recognized and counted in filaments both in soil microcosms and in liquid culture (Figure. 2.1), and the identity of these filaments verified by analyzing probe-conferred signals (Figure. 2.1).

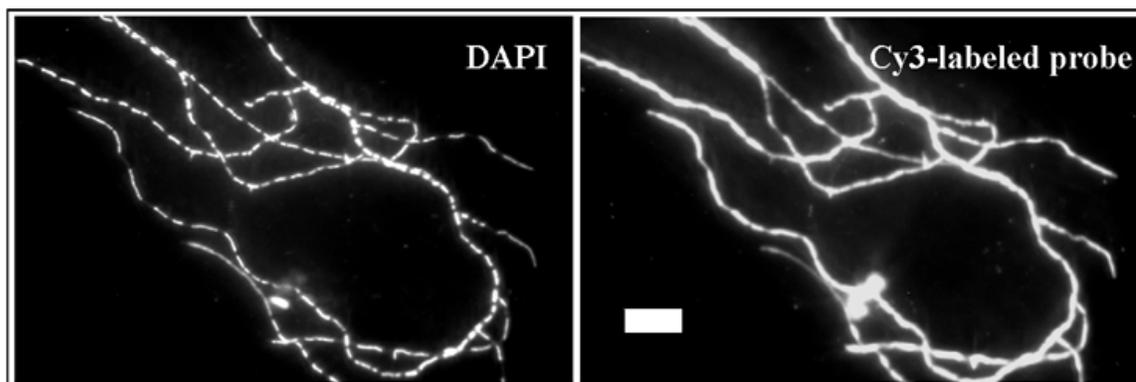


Figure 2.1: *Frankia* strain ArI3 in basal mineral medium. Detection of filaments of *Frankia* strain ArI3 in basal mineral medium (i.e., DPM without C- and N-sources) amended with 1% ground leaf litter of *Alnus glutinosa* after DAPI staining (left) and concomitant *in situ* hybridization with Cy3-labeled probe 23ArI3 (right). Bar represents 30 μm .

In situ hybridization resulted in bright signals on cells of both *Frankia* strains directly after inoculation (Figure. 2.2). For strain Ag45/Mut15, these signals remained bright for all cells in the entire filament that increased in length until about 6 to 8 weeks of incubation (Figure. 2.2). Afterwards, only parts of the filaments hybridized showing bright signals. Non-hybridized parts were still detectable by DAPI staining. These filaments did not break into fragments over time. Similar results were obtained for strain ArI3, however, non-hybridized parts of the filaments were already detected at the first sampling after 2 weeks of incubation (Figure. 2.2). After this time, bright signals of hybridized cells were detected only occasionally.

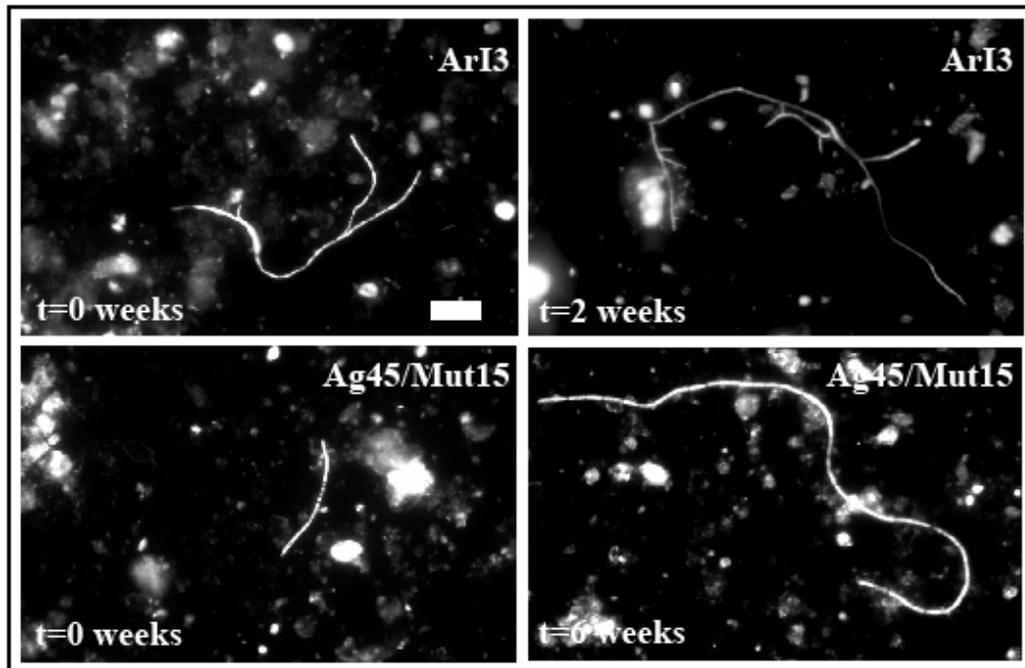


Figure 2.2: *Frankia* strains ArI3 and Ag45/Mut15 in soil amended with leaf litter of *Alnus glutinosa*. Detection by in situ hybridization of filaments of *Frankia* strains ArI3 (upper panel) and Ag45/Mut15 (lower panel) in soil microcosms amended with 5% ground leaf litter of *Alnus glutinosa*, directly after inoculation (t=0 weeks) or after incubation (t=2 weeks for strain ArI3, and t=6 weeks for strain Ag45/Mut15). Bar represents 30 μm .

Image analysis of DAPI-stained or hybridized cells of *Frankia* strains Ag45/Mut15 and ArI3 inoculated into leaf-litter amended soil showed significant differences between strains (Figure. 2.3). Numbers of filaments of strain Ag45/Mut15 inoculated at a density of about 3×10^4 cells (g soil dry wt.)⁻¹ did not change significantly during the 12-week incubation both in individual and mixed cultures, even though average filament numbers declined after about 6 to 8 weeks of incubation (Figure. 2.3). Numbers of filaments of strain ArI3, however, inoculated at a similar density declined within 4 weeks to levels below the detection limit in both individual and mixed cultures (Figure. 2.3). This decline

in numbers of filaments was accompanied by comparable declines in filament length and cell numbers that were below detection limit after 4 weeks of incubation (Figure. 2.3). The length of filaments of strain Ag45/Mut15, however, increased significantly during incubation, e.g., from about 100 μm at the time of inoculation to about 500 μm after 6 weeks, with increments being more pronounced in individual than in mixed cultures. Highest values for filament length were obtained later in individual cultures (i.e., after 6 weeks of incubation) than in mixed cultures (4 weeks of incubation) and were followed by declines that, however, were only statistically significant in mixed cultures (Figure. 2.3). Increases or decreases in filament length were accompanied by corresponding gains or losses in cell numbers (Figure. 2.3) which resulted in relatively constant average cell lengths for both strains in time, i.e., a cell length of 6 - 7.5 μm .

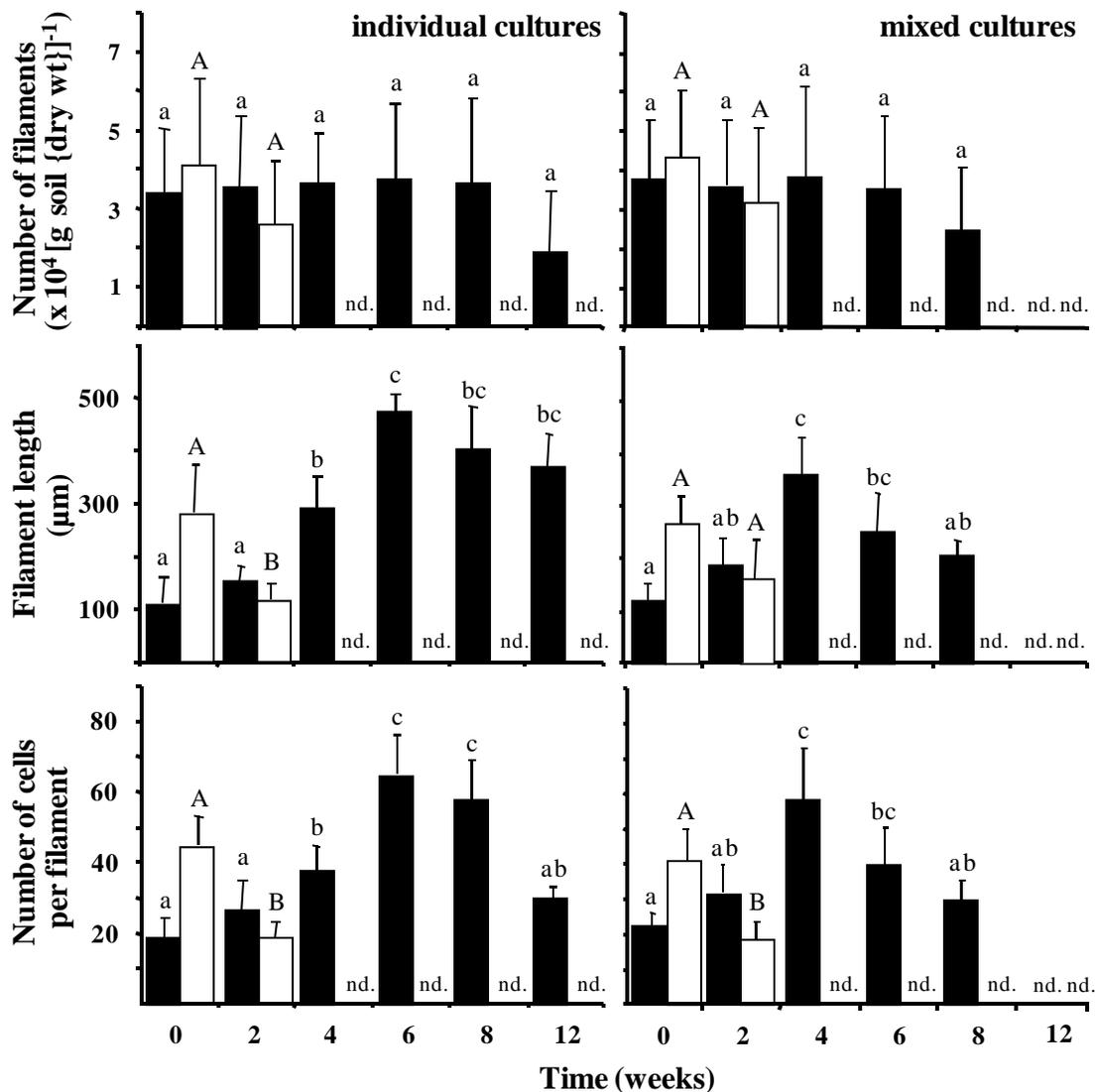


Figure 2.3: Growth characteristics of *Frankia* strains Ag45/Mut15 and ArI3 in soil microcosms amended with leaf litter of *Alnus glutinosa*. Growth characteristics (i.e., numbers of filaments, filament lengths, and number of cells per filament) of *Frankia* strains Ag45/Mut15 (closed bars) and ArI3 (open bars) in soil microcosms amended with 5% ground leaf litter of *Alnus glutinosa*. Strains were inoculated as individual cultures (left panel) or mixed (right panel) and analyzed after 0, 2, 4, 6, 8, and 12 weeks of incubation. Values are presented as mean \pm standard error (SE) (n=3). Different letters assigned to bars of the same treatment represent statistically significant differences in time (Tukeys HSD test, $P < 0.05$). “nd.” represents values that were below the detection limit.

For strain Ag45/Mut15, similar results were obtained in leaf-litter amended mineral medium (Figure. 2.4) where numbers of filaments did not significantly change during the incubation period of 12 weeks, and filament length and number of cells increased in time followed by a slight decrease towards the end of the incubation period in individual and mixed culture (Figure. 2.4). Results, however, were different for strain ArI3. In contrast to amended soil, filaments of strain ArI3 remained visible during the entire study with numbers comparable to those of strain Ag45/Mut15 (Figure. 2.4). Filament length, however, still decreased in time, as did numbers of cells both for incubation in individual or mixed cultures (Figure. 2.4). Ratios between filament length and cell numbers again resulted in average cell lengths of 6 - 7.5 μm for each strain during the entire incubation period.

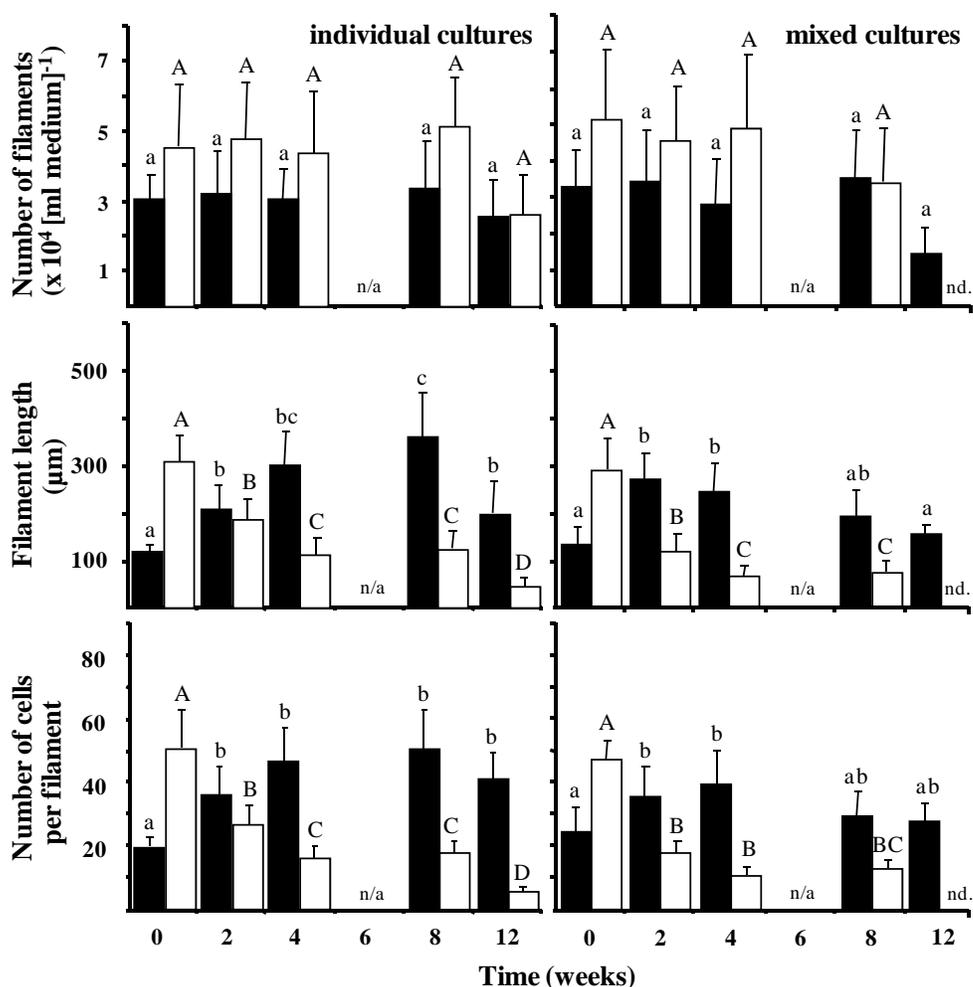


Figure 2.4: Growth characteristics of *Frankia* strains Ag45/Mut15 and ArI3 in basic mineral medium. Growth characteristics (i.e., numbers of filaments, filament lengths, and number of cells per filament) of *Frankia* strains Ag45/Mut15 (closed bars) and ArI3 (open bars) in basic mineral medium (i.e., DPM without C- and N-sources) amended with 1% ground leaf litter of *Alnus glutinosa*. Strains were inoculated as individual cultures (left panel) or mixed (right panel) and analyzed after for 0, 2, 4, 8, and 12 weeks of incubation. No samples were available for analyses after 6 weeks (n/a). Values are presented as mean \pm standard error (SE) (n=3). Different letters assigned to bars of the same treatment represent statistically significant differences in time (Tukeys HSD test, $P < 0.05$). “nd.” represents values that were below the detection limit.

Results of image analyses, i.e., increases or decreases in filament length and cell numbers were matched by rRNA content measurements for both strains in individual and mixed cultures (Figure. 2.5). For strain Ag45/Mut15, rRNA contents increased initially in amended soil as well as in mineral medium and then decreased towards the end of the incubation period, while those for strain ArI3 decreased during the entire incubation period. Similar to results of image analyses for filament length and number of cells, rRNA representing strain ArI3 decreased to concentrations below the detection limit within 4 weeks in soil, but remained detectable until 8 weeks after incubation in amended mineral medium (Figure. 2.5). Values for rRNA contents were generally about twice as high in mineral medium compared to soil due to higher nucleic acid extraction efficiencies in mineral medium. This methodological artifact affects calculated average values for rRNA contents per cell that were consequently higher in mineral medium (about 10 – 12 fg per cell) than in soil (4 – 9 fg per cell).

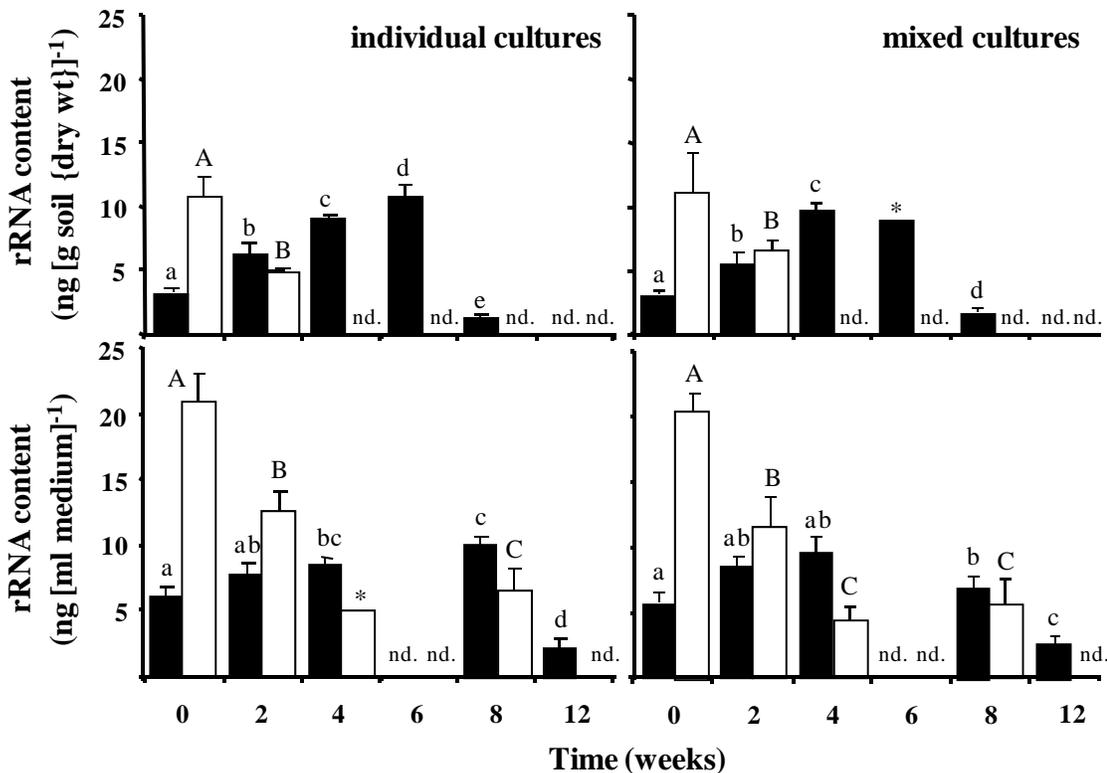


Figure 2.5: rRNA contents of *Frankia* strains Ag45/Mut15 and ArI3 in soil amended with 5% ground leaf litter of *Alnus glutinosa*, and in basic mineral medium. rRNA contents of *Frankia* strains Ag45/Mut15 (closed bars) and ArI3 (open bars) in soil microcosms amended with 5% ground leaf litter of *Alnus glutinosa* (upper row), and in basic mineral medium (i.e., DPM without C- and N-sources) amended with 1% ground leaf litter of *Alnus glutinosa* (lower row). Strains were inoculated as individual cultures (left panel) or mixed (right panel) and analyzed after 0, 2, 4, 6, 8, and 12 weeks of incubation. Values are presented as mean \pm standard error (SE) (n=3). Different letters assigned to bars of the same treatment represent statistically significant differences in time (Tukey's HSD test, P<0.05). "*" indicates the absence of replicate samples, and "nd." values that were below the detection limit.

Discussion

In situ hybridization and concomitant DAPI staining have been shown to be adequate tools for the quantitative analysis of microorganisms in both aquatic and terrestrial environments in the past (Amann *et al.*, 1995; Amann *et al.*, 1997; Hahn & Zeyer, 1994).

These tools have successfully been used to identify specific *Frankia* strains in root nodules, and to detect them after introduction into soil (Hahn *et al.*, 1997; Hahn *et al.*, 1999). Their usefulness, however, depended on pretreatments enhancing the permeability of cells for probes, and on detection systems that reliably distinguished between probe-conferred and autofluorescent signals (Hahn *et al.*, 1993; Zepp *et al.*, 1997a). Although the basic methodology for this qualitative analysis of frankiae in the environment has been available for some time, it has not been used for quantitative analyses of frankiae in the environment so far.

Our approach to quantify *Frankia* cells in the environment by *in situ* hybridization was based on pretreatments with lysozyme, followed by achromopeptidase as suggested for actinomycetes (Sekar *et al.*, 2003). These pretreatments allowed us to hybridize and detect cells of both strains reliably (Figure. 2.1, 2.2). DAPI staining carried out concomitantly during hybridization, however, proved to be superior over *in situ* hybridization for our purpose of quantification since it provided clearer images of individual cells within filaments, and thus measurements of filament length and cell numbers were more accurate than using hybridization (Figure. 2.1). Since the morphology of the introduced *Frankia* strains was quite distinctive compared to those of other genera in the soil, analyses of DAPI-stained cells could have provided information on growth characteristics of both strains in individual culture. *In situ* hybridization, however, was necessary to distinguish between *Frankia* strains and thus to quantify each strain in mixed culture.

An additional advantage of using *in situ* hybridization over DAPI-staining was that rRNA was targeted instead of DNA. The cellular rRNA content provides some indication of the

physiological state of the cell (Hood *et al.*, 1986; Kramer & Singleton, 1992) and has been correlated with the growth rate of fast growing bacteria (DeLong *et al.*, 1989; Kramer & Singleton, 1992). This correlation, however, may not be valid during slow growth and starvation (Chapman & Gray, 1981; Kemp *et al.*, 1993; Tolker Nielsen *et al.*, 1997). In our study, cell numbers determined by hybridization matched those obtained after DAPI staining for both strains only directly after inoculation (data not shown). This relationship had changed for strain ArI3 already at the first sampling after 2 weeks of incubation. At this time all cells were still stained with DAPI, however, only a few cells were hybridized indicating large reductions in rRNA content in those cells that did not hybridize. Reductions in rRNA content to between 30 and 50% of the amount encountered in vegetative cells often accompanies the formation of resting cells or spores by which many bacteria adapt to adverse environmental conditions (Givskov *et al.*, 1994; Quiros *et al.*, 1989; Siala *et al.*, 1974). Our rRNA content analyses both in soil and in mineral medium showed larger reductions, e.g., a reduction from 10 ng (g soil { dry wt.})⁻¹ to below the detection limit which is lower than 1 ng (g soil { dry wt.})⁻¹. Values below 10% of the original rRNA content have been related to a loss of viability of cells (Davis *et al.*, 1986) or to physiologic changes negatively affecting viability (Kaplan & Apirion, 1975; Kramer & Singleton, 1992). The decrease in *in situ* hybridization signals on cells of strain ArI3 during the initial phases of the incubation, i.e., after 2 weeks in soil, and for strain Ag45/Mut15 towards the end of the incubation period, i.e., after 8 weeks in soil, correlates well with rRNA content profiles, and therefore suggests reductions in viability of cells of both strains at different time steps during the incubation. Proof of this suggestion, however, requires additional studies that attempt to resuscitate

both strains after declines in rRNA contents, and observe potential changes in detectability of cells by *in situ* hybridization.

In situ hybridization allowed us to show that filaments of *Frankia* strain Ag45/Mut15 introduced into soil and mineral medium amended with ground leaf litter increased in length and that this increment was related to concomitant increments in cell numbers. These results demonstrate that cells of this strain proliferate in the presence of leaf litter as sole C- and N-source, and therefore show their potential to grow saprophytically in the environment. Declines of cell numbers and rRNA content towards the end of the incubation period might be the consequence of reductions in resource availability which is an assumption that could be proven by the addition of leaf litter just before these declines occur after 6 to 8 weeks of incubation.

This situation is different for strain ArI3 that did not grow after inoculation, even though growth of strain Ag45/Mut15 alone and in mixed culture suggested sufficient nutrient resource availability. Since many basic environmental conditions resembled those for pure cultures (e.g., inorganic nutrients, pH, temperature), and our studies on growth of inoculated *Frankia* strains ArI3 and Ag45/Mut15 in sterilized amended mineral medium provided the same outcome as the studies in non-sterile soil microcosms, many abiotic and biotic factors leading to soil microbiostasis (van Veen *et al.*, 1997) can be ruled out as potential causes for the decline in population size of strain ArI3. The most likely candidate of factors affecting growth of strain ArI3 is therefore carbon resource availability. Strain ArI3 might not be able to grow on complex organic material such as leaf litter, but require easily available C-resources represented by, for example, root exudates (Rönkkö *et al.*, 1993; Smolander, 1990a). This speculation is supported by

results of previous studies where root nodule forming populations entirely represented by frankiae similar to strain ArI3 in the original soil were shifted to populations entirely represented by strain Ag45/Mut15 after leaf litter amendment to soil and incubation for 6 weeks (Nickel *et al.*, 2001). Inoculation with both strains ArI3 and Ag45/Mut15 into amended or non-amended soil and subsequent incubation did not change this outcome significantly (Nickel *et al.*, 2001). In order to support this speculation, a similar set of experiments as presented here, but with plants in exchange for leaf litter amendments could be performed to retrieve information on this requirement for strain ArI3, and to exclude a potential alternative explanation that strain ArI3 is sensitive to inoculation where transfer from nutrient-rich liquid media to nutrient limited or poor environments results in a rapid decline of viability (van Veen *et al.*, 1997).

Acknowledgements

This study was financially supported by a grant from the Research Enhancement Program (REP) at Texas State University-San Marcos.

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CHAPTER III

GROWTH OF *FRANKIA* STRAINS IN LEAF LITTER-AMENDED SOIL AND THE RHIZOSPHERE OF A NON-ACTINORHIZAL PLANT

Abstract

The ability of *Frankia* strains to grow in the rhizosphere of a non actinorhizal plant, *Betula pendula*, in surrounding bulk soil and in soil amended with leaf litter was analyzed six weeks after inoculation of pure cultures by *in situ* hybridization. Growth responses were related to taxonomic position as determined by comparative sequence analysis of *nifH* gene fragments and of an actinomycetes-specific insertion in Domain III of the 23S rRNA gene. Phylogenetic analyses confirmed the basic classification of *Frankia* strains by host infection groups, and allowed a further differentiation of *Frankia* clusters within the *Alnus* host infection group. Except for *Casuarina*-infective *Frankia* strains, all other strains of the *Alnus* and the *Elaeagnus* host infection groups displayed growth in the rhizosphere of *B. pendula*, and none of them grew in the surrounding bulk soil that was characterized by very low organic matter content. Only a small number of strains that all belonged to a distinct phylogenetic cluster within the *Alnus* host infection group, grew in soil amended with ground leaf litter from *B. pendula*. These results demonstrate that saprotrophic growth of frankiae is a common trait for most members of the genus, and the

supporting factors for growth (i.e. carbon utilization capabilities) varied with host infection group and phylogenetic affiliation of the strains.

Key words: fluorescent *in situ* hybridization (FISH), microcosms, probes, saprotrophic growth

Introduction

Actinorhizal plants are characterized by their ability to form root nodules in symbiosis with nitrogen fixing actinomycetes of the genus *Frankia* (Akkermans *et al.*, 1992).

Because actinorhizal plants can get up to 90 percent of their nitrogen requirement from this symbiotic association (Domenach *et al.*, 1989), they are uniquely successful pioneer plants that frequently establish themselves on nutrient-limited or degraded sites including those impacted by catastrophic events (Dawson, 1986). In silviculture, actinorhizal plants such as *Alnus* or *Elaeagnus* species are therefore often used for land reclamation and in habitat restoration efforts (Dawson, 1986; Roy *et al.*, 2007). Although frankiae have been shown to be present in disturbed or impacted soils (Huss-Danell & Frej, 1986; Ridgway *et al.*, 2004; Yamanaka & Okabe, 2006), inoculation of actinorhizal plants with *Frankia* strains, usually in fumigated nursery soils with subsequent transplantation to the impacted sites, is a recommended practice to increase chances for their successful establishment in these soils (McEwan *et al.*, 1999; Quoreshi *et al.*, 2007; Wheeler *et al.*, 1991).

Many studies have shown positive effects of inoculation on plant establishment and subsequent growth performance (Hilger *et al.*, 1991; Houwers & Akkermans, 1981; Wheeler *et al.*, 1986); little, however, is known about the establishment of inoculated strains in root nodules and in soils. Only a few studies have demonstrated that inoculated strains successfully competed with indigenous populations (Nickel *et al.*, 1999; Nickel *et*

al., 2001) or with other inoculated strains for nodule formation (Martin *et al.*, 2003), and that they can persist and remain infective in soils for some time (McEwan *et al.*, 1999; Smolander *et al.*, 1988b). It is unknown, however, whether nodule formation is initiated by frankiae actively growing in soil or whether dormant stages such as spores that persist long-term in the environment are activated by the host plant.

The potential for both active growth as well as for long-term persistence of frankiae in soil was demonstrated for both inoculated and indigenous populations when leaf litter amendment to soil characterized by very low organic matter content and incubation for 6 weeks before planting resulted in extreme differences in nodule-forming *Frankia* populations on *Alnus glutinosa* compared to those in soil left non-amended (Nickel *et al.*, 2001). This analysis was based on the differentiation of two subgroups within the *Alnus* host infection group using sequence differences in an actinomycetes-specific insertion in Domain III of the 23S rRNA gene of *Frankia* strains as taxonomic criterium (Hönerlage *et al.*, 1994). Inoculation of two *Frankia* strains ArI3 and Ag45/Mut15 representing the two subgroups into leaf-litter amended and non-amended soils and incubation for 6 weeks before planting resulted in the establishment of both strains in nodules of plants grown on non-amended soil, while frankiae in nodules on plants grown on leaf-litter amended soils were almost entirely represented by strain Ag45/Mut15 (Nickel *et al.*, 2001). Follow-up studies demonstrated saprotrophic growth on leaf litter for strain Ag45/Mut15, but not for strain ArI3 (Mirza *et al.*, 2007).

The aim of this study was to test whether saprophytic growth of frankiae is a common trait for members of the genus, but more likely on easily available carbon (C) sources such as root exudates than on complex organic matter such as leaf litter. Saprotrophic

growth was tested in microcosms with leaf-litter amended soils and non-amended soils vegetated with *Betula pendula* that were inoculated with individual *Frankia* strains. Most of these strains belonged to the *Alnus* host infection group (n=27), but also included *Casuarina*-infective strains (n=3), that are a phylogenetically distinct group within the *Alnus* host infection group, and strains of the *Elaeagnus* host infection group (n=4). Growth was analyzed after 6 weeks of incubation by *in situ* hybridization (Mirza *et al.*, 2007), and related to taxonomic groups that were compiled by comparative sequence analyses of *nifH* gene fragments (Mirza *et al.*, 2009; Welsh *et al.*, 2009) and of the actinomycetes-specific insertion in Domain III of the 23S rRNA gene (Hönerlage *et al.*, 1994; Roller *et al.*, 1992).

Material and Methods

Experimental setup: Microcosms were established in 2-ml cryotubes that contained 1 g of air-dried, unsterile soil obtained from a natural stand of *A. glutinosa* (Ettiswil, Switzerland) (Zepp *et al.*, 1997b) and characterized by a low content of organic material (0.02%) (Nickel *et al.*, 2001). Half of the tubes were vegetated with three seedlings of *Betula pendula* Roth (European White Birch), a non-host plant for frankiae. Seeds (Lawyer Nursery, Inc., Plains, MT) were surface-sterilized in 30% H₂O₂ for 10 minutes and two subsequent washes in distilled sterilized water, and germinated on water agar. Two-week-old seedlings were transferred to soil in cryotubes and grown for 6 weeks, fertilized once a week with approx. 1 ml of a 25% Hoagland solution (Zimpfer *et al.*, 1997). The second half of the tubes remained non-vegetated, but soil was amended with 5% (wt/wt) leaf litter of *B. pendula* ground to a particle size of about 0.1 mm (Mirza *et al.*, 2007).

Six weeks after transplantation, microcosms with plants and those with leaf-litter amended soil were inoculated with *Frankia* strains (n=34, Table 1) that were grown in Defined Propionate Medium (DPM) containing propionate and NH₄Cl as C and N source, respectively (Meesters *et al.*, 1985) for two weeks. Cultures were harvested by centrifugation (15,000 x g, 5 minutes), washed with distilled water twice, and cell aggregates disrupted in basal DPM mineral medium without C and N sources using a tissue homogenizer. Homogenized cultures were diluted to a density of about 10⁷ cells ml⁻¹ in basal DPM mineral medium, and inoculated in a final volume of 500 µl. Microcosms with amended soils were incubated at 30°C in the dark, while those with plants were kept at 20°C on a 16:8 hour light:dark schedule. Microcosms were sampled destructively after inoculation (t=0) and after 6 weeks of incubation (t=6) (n=3 tubes per treatment, strain and time) (Mirza *et al.*, 2007). Controls included non-inoculated microcosms, both for vegetated and leaf-litter amended microcosms, to confirm the lack of detection of indigenous frankiae.

Sample preparation: Soils in leaf-litter amended microcosms were directly fixed in cryotubes with 4% paraformaldehyde in phosphate buffered saline (PBS; 0.13 M NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, pH 7.2) at 4°C for 16 hours (Hahn *et al.*, 1992), while in vegetated microcosms rhizosphere soil was separated from bulk soil before fixation. Rhizosphere soil was defined as the soil adhering to roots after removal of the plants from soil, with excess soil shaken off the roots. Bulk soil was defined as all remaining soil, not adhering to the roots. After fixation rhizosphere soil was washed off the plant roots which were then discarded. All samples were subsequently washed in PBS twice

and stored in a final volume of 1 ml of 50% ethanol in PBS at -20°C until further use (Amann *et al.*, 1990a).

Growth analysis: *In situ* hybridization was used to assess growth of *Frankia* strains (i.e., increase in filament length) in 10 µl sub-samples that were diluted in 90 µl of 0.1 % sodium pyrophosphate. Ten µl of these dilutions were spotted onto gelatin coated slides (0.1% gelatin, 0.01% KCr(SO₄)₂), dried at 42°C for 15 minutes, and subsequently dehydrated in 50, 80 and finally 96% ethanol for three minutes each. Cells were then treated with 10% dimethylsulfoxide (DMSO) for 30 minutes to enhance their permeability (Taniuchi *et al.*, 2008). Slides were rinsed with distilled water and dehydrated again in 50, 80, and 96% ethanol for three minutes each.

Samples were hybridized with Cy3-labeled probe EUB338 (5' GCT GCC TCC CGT AGG AGT) that binds to the 16S rRNA of many bacteria including frankiae (Amann *et al.*, 1990b). Detection of frankiae with this probe was based on the distinctive morphology of frankiae (i.e. filaments and very large cell size [$>5\mu\text{m}$ in length] compared to the indigenous community that was generally single celled and very small [$<1\ \mu\text{m}$], and the lack of tertiary branching generally found in other actinomycetes such as *Streptomyces* sp.). These analyses were verified on selected strains by hybridization with probes 23ArI3 (5' CCA GAC ACA TCT CCG AA) or 23Mut(II) (5' CCA CAC ACA CCC CCT AA) (Zepp *et al.*, 1997a) targeting *Frankia* subgroup-specific sequences on an insertion in Domain III of the 23S rRNA specific for actinomycetes (Roller *et al.*, 1992). Hybridizations were performed in 9 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, 5 mM EDTA, 0.01% SDS, pH 7.2) containing of 30% formamide, 1 µl of

probe ($25 \text{ ng } \mu\text{l}^{-1}$) and $1 \text{ } \mu\text{l}$ of the DNA-intercalating dye 4',6-diamidino-2-phenylindole (DAPI, final concentration $200 \text{ ng } \mu\text{l}^{-1}$) at 42°C for 2 hours.

After hybridization, slides were washed with hybridization buffer at room temperature for 15 minutes, rinsed with distilled water and air-dried. Slides were mounted with Citifluor AF1 solution (Citifluor Ltd., London, UK) and examined with a Nikon Eclipse 80i microscope, fitted for epifluorescence microscopy with a mercury lamp (X-Cite™ 120; Nikon) and two filter cubes, UV-2E/C (Nikon; EX340-380, DM400, BA4435-485, for DAPI detection) and Cy3 HYQ (Nikon; EX535/50, DM565, BA610/75, for Cy3 detection), respectively. DAPI and Cy3 pictures were taken from the same image using a digital camera (DXM 1200F; Nikon), and 25 images per treatment, time and replicate were analyzed for filament length using Nikon's NIS Elements imaging software (Version 3). Analyses of filament length provided qualitative information on growth or non-growth of strains under different environmental conditions, but could not be used for quantitative comparisons of growth rates among strains because initial filament lengths and cell sizes of individual strains were not identical.

Sequence analyses: One ml samples of homogenized aggregates of pure cultures of *Frankia* remaining after inoculation of microcosms were centrifuged, and pellets re-suspended in 95 μ l of distilled water. Suspensions were mixed with 5 μ l of proteinase K solution (Promega, Madison, WI, 30 U mg^{-1} ; 10 mg ml^{-1} in water) and incubated at 37°C for 20 minutes. Then, 0.5 μ l of 10% SDS solution was added and the mixture incubated at 37°C for another 3 hours which was followed by a final incubation at 80°C for 20 minutes.

NifH gene fragments (606 bp) were amplified using primers *nifHf1* (5' GGC AAG TCC ACC ACC CAG C) and *nifHr* (5' CTC GAT GAC CGT CAT CCG GC) (Mirza *et al.*, submitted; Welsh *et al.*, 2009) in a reaction volume of 50 μ l, containing 1 μ l of a 10 mM dNTP mix, 0.5 μ l each primer (0.4 μ M), 8.2 μ l BSA (30 $\mu\text{g ml}^{-1}$), 5 μ l of 10 x PCR buffer with 15 mM MgCl_2 , 2 μ l of pure culture lysate, and 0.2 μ l *Taq* DNA polymerase (5 U μ l⁻¹; Gene Script, Piscataway, NJ) that was added after an initial incubation at 96°C for 10 minutes. The addition of *Taq* polymerase was followed by 35 rounds of temperature cycling (96°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds) and a final 7 minute incubation at 72°C. Fragments containing the actinomycete-specific insertion in Domain III of the 23S rRNA gene (approx. 150 bp) were amplified using primers 23InsVFra (5' CAG GCG TAG TCG ATG G) and 23Fra (5' ATC GCA TGC CTA CTA CC) (Hönerlage *et al.*, 1994) using the same conditions as described above, except for temperature cycling conditions (96°C for 30 seconds, 54°C for 30 seconds and 72°C for 30 seconds). Sub-samples of the reactions (5 μ l) were checked for amplification products by gel electrophoresis (1% agarose in Tris-acetate-EDTA (TAE) buffer, wt/vol) after staining with ethidium bromide (0.5 $\mu\text{g ml}^{-1}$) (Sambrook *et al.*, 1989).

Amplified *nifH* gene and 23S rRNA gene insertion fragments were purified using the Ultra Clean 15 DNA Purification Kit (MoBio, Carlsbad, CA), and sequenced using the CEQ 8800 Quickstart Kit according to the manufacturer's instructions (Beckman Coulter, Fullerton, CA) with the addition of 5% DMSO to the reaction mix. The sequencing reaction consisted of an initial incubation at 76°C for 5 minutes followed by 76°C for 5 minutes during which primer and master mix were added, a subsequent incubation at 94°C for 2 minutes, 35 cycles of temperature cycling (94°C for 30 seconds, 50°C for 30 seconds and 60°C for 4 minutes) and a final extension at 60°C for 10 minutes (Kukanskis *et al.*, 1999). Sequences were analyzed on a CEQ 8800 sequencer (Beckman Coulter), and deposited at Genbank under accession numbers FJ477419-FJ477438 and FJ483975-FJ484007.

Phylogenetic analyses: Amplified *nifH* gene and 23S rRNA gene insertion fragments were trimmed to be 552 bp and 146 bp long, respectively. Both genes for each of the 34 strains were aligned in two different data sets using Sequencher 4.2.2 (Gene Codes Corp., Ann Arbor, MI), CLUSTAL X, and MacClade 4.05 (Maddison & Maddison, 1999; Thompson *et al.*, 1997) and analyzed using maximum parsimony (MP), neighbor joining (NJ), Bayesian, and maximum likelihood (ML) methods. The outgroup for each analysis was *Frankia* strain EAN1pec.

MP methods began by using MacClade 4.05 to chart the rate of mutation by codon position. Third position changes of the *nifH* gene were down-weighted and thus first position changes up-weighted to better delineate meaningful changes (i.e. in protein structure) between these sequences. The 23S rRNA gene insertion data set was not weighted at all. MP analyses were completed in the program PAUP*4.0b10 (Swofford,

2002), and included 10,000 heuristic random addition replicates, tree bisection–reconnection (TBR), and “no mul” trees (Swofford, 2002). Confidence in the topology for these MP trees was gauged using bootstrap re-sampling methods (BS) in PAUP* and included 10,000 replications and a full heuristic search (Felsenstein, 1985). Only those BS values of at least 70% demonstrate good support measures and thus were retained (Hillis & Bull, 1993).

NJ methods were also used in PAUP*. Modeltest version 3.7 was used to determine the model of sequence evolution that fit each data set best (Posada & Crandell, 1998). For the *nifH* gene, a general time reversible (GTR) model was selected and specific values for the gamma shape parameter and proportion of invariant sites provided by Modeltest were entered under the distance settings for the NJ GTR model in PAUP*. For the 23S rRNA gene insertion data set the Jukes Cantor (JC) model was selected. The BS test in PAUP* included 10,000 replications and a neighbor joining search.

Bayesian analyses for each dataset were completed using MRBAYES version 3.0 and included Metropolis-coupled Markov chain Monte Carlo (MCMCMC) sampling. The *nifH* gene dataset used a GTR+I+G model of sequence evolution estimated during the run and the 23S rRNA gene insertion dataset used a JC model. Settings for both data sets included 5 million generations and sampling every 1000 trees (Huelsenbeck & Ronquist, 2001). 95% majority rule consensus trees for the Bayesian output of posterior probabilities (PP) was created in PAUP* with the first 10 trees from each data set removed as burn-in (Huelsenbeck *et al.*, 2002).

Maximum likelihood analyses were completed using the RAxML-VI-HPC program (Stamatakis, 2006b) on the computer cluster of the ‘CyberInfrastructure for Phylogenetic

RESearch' project (CIPRES, www.phylo.org) from the online servers at the San Diego Supercomputing Center. Settings GTR+CAT approximation for rate heterogeneity (Stamatakis, 2006a), invariant sites, empirical base frequencies and estimation of the necessary number of bootstrap replicates were used.

Results and discussion

Reliable detection of frankiae by *in situ* hybridization required pretreatment with DMSO for 30 minutes. This treatment enabled us to detect all cells inoculated into soil directly after inoculation, but only for a subset of strains and treatments after incubation for 6 weeks. Concomitant DAPI-staining, however, detected cells of all *Frankia* strains independent of treatment, time and the absence or presence of probe-conferred signals (data not shown). Morphologies of cells detected with probe EUB338 corresponded to those detected with *Frankia* specific probes on selected strains (Ar13 and Ag45/Mut15). Non-inoculated control soils did not result in the detection of cells showing the typical morphologies of frankiae after inoculation. Basic morphologies of cells or filaments of those strains detected after incubation did not differ noticeably from those directly after inoculation, except for changes in filament length (Figure. 3.1). Filaments increasing in length were detectable by *in situ* hybridization and DAPI-staining, while those that did not increase in length were only detectable after DAPI-staining. These results indicate a reduction of target molecules (i.e. rRNA) in non-growing strains while those that were growing maintained concentrations that were adequate for detection by *in situ* hybridization.

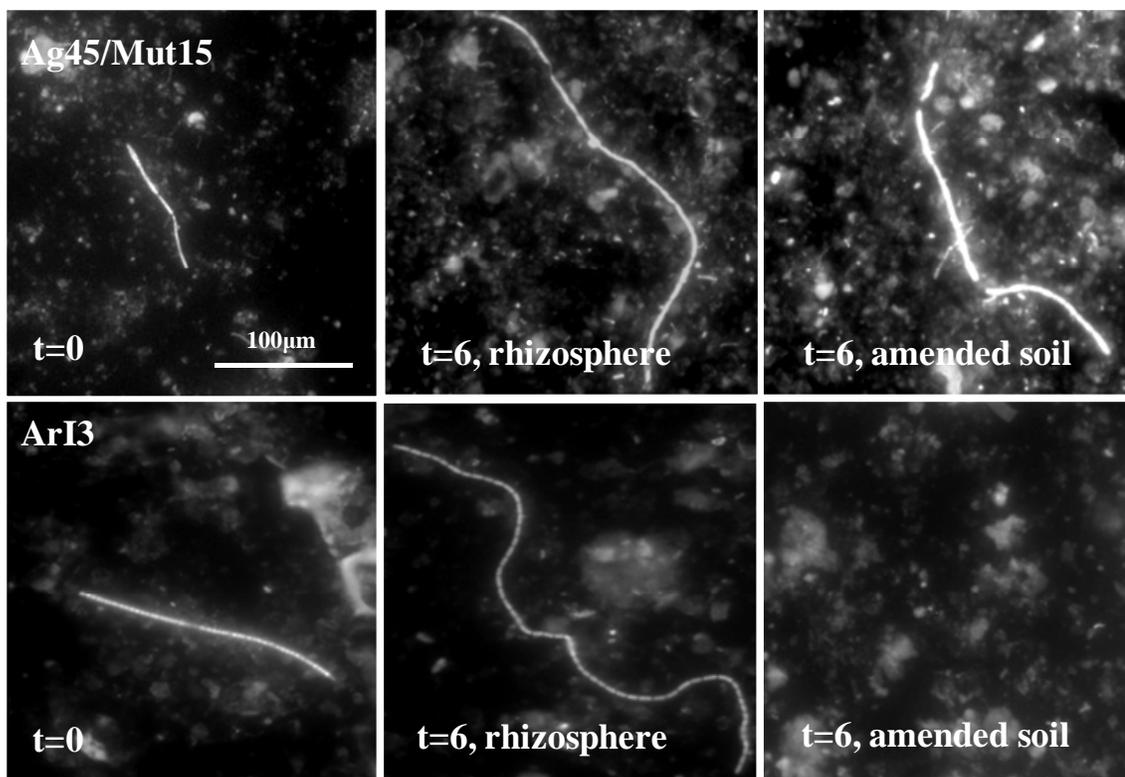


Figure 3.1: *Frankia* strains Ag45/Mut15 and ArI3 in soil and rhizosphere of *Betula pendula*. Detection by *in situ* hybridization of filaments of *Frankia* strains Ag45/Mut15 (upper panel) and ArI3 (lower panel), directly after inoculation into soil microcosms (t=0), or after incubation for 6 weeks in microcosms with soil either vegetated with *Betula pendula* (t=6, rhizosphere) or amended with 5% ground leaf litter of *B. pendula* (t=6, amended soil). Bar represents 100 μ m.

Ribosomal RNA contents per cell have been used as an indicator of the physiological state of cells, with rRNA contents directly correlated to growth rate in fast growing organisms (Kramer & Singleton, 1992a). Although a direct correlation of rRNA content and physiological state might not be established for slow growing organisms or during starvation (Kemp *et al.*, 1993; Nielsen *et al.*, 1997), and thus not be valid for members of the slow-growing actinomycete *Frankia*, higher rRNA contents were found in growing *Frankia* strains and those detectable by *in situ* hybridization in our previous study (Mirza *et al.*, 2007). Thus, increased filament length and detection by *in situ* hybridization after

incubation in our study demonstrates growth and the presence of metabolically active cells of frankiae in the respective treatment.

When rhizosphere samples were analyzed by *in situ* hybridization, increased filament length by factors between two and three were found in all 27 strains belonging to the *Alnus* host infection group, as well as in the four strains tested that belonged to the *Elaeagnus* host infection group (Figure. 3.2). Strains CeF, CjI-82, and CcI3, however, representing *Casuarina*-infective frankiae, could not be visualized by *in situ* hybridization in rhizosphere samples after 6 weeks of incubation (Figure. 3.2). None of the 34 strains could be detected by *in situ* hybridization in bulk soil. These results corroborate those of previous studies where growth of *Frankia* was found in the rhizosphere of host and non-host plants under axenic conditions, most likely influenced by root exudates that are easily available C sources (Rönkkö *et al.*, 1993; Smolander *et al.*, 1990). The assumption that root exudates are readily available C sources is supported by the detection of growing frankiae in the rhizosphere only and not in the surrounding bulk soil which might be the result of a fast decreasing water soluble fraction of root exudates with increasing distance from the roots (Huetsch *et al.*, 2002).

Root exudates of birch are known to contain organic acids such as citric, adipic, propionic, succinic and acetic acid in addition to carbohydrates (Sandnes *et al.*, 2005; Smith, 1976). Propionic acid is generally used as a universal C source for growth of *Frankia* in pure culture, and was used in this study as the sole C source to maintain and grow all pure cultures, including the *Casuarina*-infective strains. *Casuarina*-infective strains, however, did not grow in the rhizosphere of *B. pendula* (Figure. 3.2), even though these same strains did grow in the rhizosphere of *Casuarina cunninghamiana* (data not

shown). The latter is consistent with results of previous studies that demonstrated positive effects of tissue and roots of *Casuarina* plants on growth of *Casuarina*-infective *Frankia* strains (Zimpfer *et al.*, 2004). Our results demonstrate that frankiae of the *Alnus* and *Elaeagnus* host infection groups can grow saprotrophically in the rhizosphere of a non-host plant, most likely due to the availability of adequate C sources, while *Casuarina*-infective *Frankia* strains require more complex conditions for growth provided only by the host plant. The nature of these conditions, however, does not include a very particular carbon source requirement.

In contrast to results obtained for the rhizosphere of *B. pendula*, saprotrophic growth on leaf litter as a C source is restricted to a small fraction of *Frankia* strains belonging to the *Alnus* host infection group as demonstrated by increased filament lengths (Figure. 3.2). The majority of strains of the *Alnus* host infection group (i.e. 21 of 27 strains) as well as the 4 strains tested for the *Elaeagnus* host infection group and the 3 *Casuarina*-infective strains were not detectable by *in situ* hybridization six weeks after inoculation (Figure. 3.2). These results suggest that leaf litter represents an inadequate C source for most *Frankia* strains, which is most likely a consequence of the complexity of this material. Although enzymes such as cellulases (Igual *et al.*, 2001), or genes of putative polysaccharolytic enzymes (Mastronunzio *et al.*, 2008) have been detected in several *Frankia* strains, plant cell wall degrading capacity of these frankiae was suggested to be low (Mastronunzio *et al.*, 2008). Since none of the strains shown to grow in the presence of leaf litter in our study was included in any analyses on polysaccharolytic enzymes, future studies should address questions on the potential of these strains to grow on components characteristic for leaf litter such as e.g., cellulose, but also include the

assessment of potential mediating effects of indigenous microorganisms that hydrolyze complex leaf litter and might provide monomeric products for growth of these frankiae.

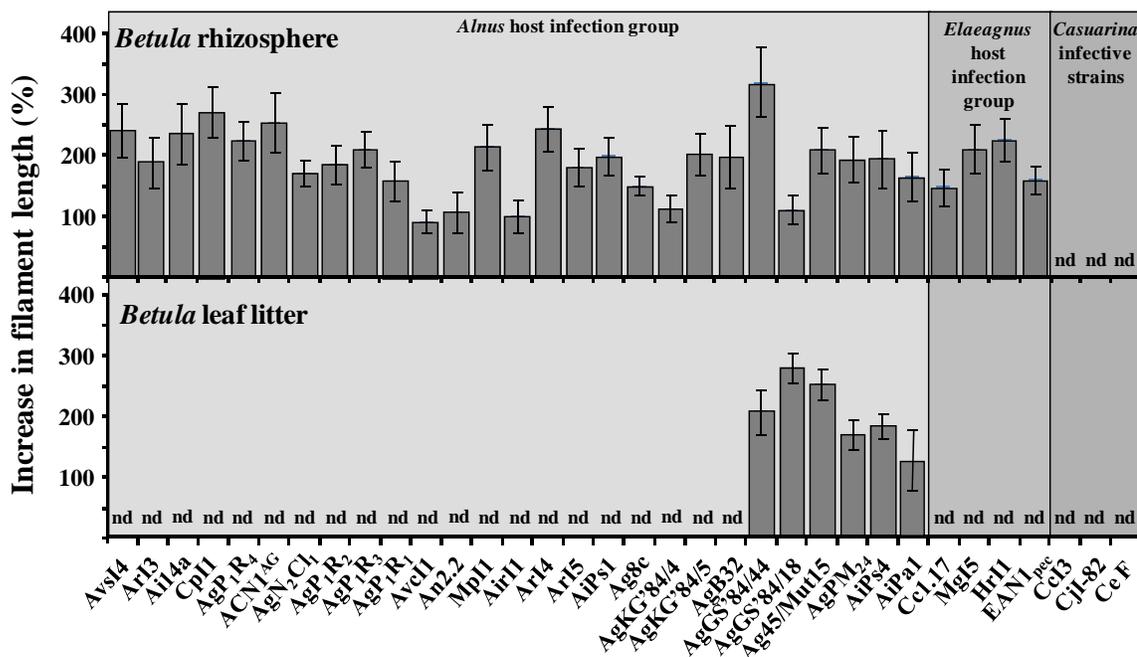


Figure 3.2: Growth of 34 *Frankia* strains in soil and rhizosphere of *Betula pendula*. Increase in filament length (%) of 34 *Frankia* strains belonging to the *Alnus* host infection group (n=27), the *Elaeagnus* host infection group (n=4) or representing *Casuarina*-infective strains (n=3), 6 weeks after inoculation into microcosms containing soil either vegetated with *Betula pendula* (birch rhizosphere, upper panel) or amended with 5% ground leaf litter of *B. pendula* (leaf litter amended soil, lower panel). The error bars represent 95% confidence intervals. nd = no growth detected.

Comparative sequence analyses of both *nifH* gene fragments and the insertion in domain III of the 23S rRNA gene resulted in a consistent grouping of *Frankia* strains on the basis of host plant specificities, i.e., they assigned *Frankia* strains into the *Alnus* or *Elaeagnus* host infection groups, and as *Casuarina*-infective strains (Figure. 3.3). These results were independent of phylogenetic analyses method used (i.e. MP, NJ, Bayesian, and ML

methods), with high BS or PP support at the major nodes (Figure. 3.3) and were consistent with classification by comparative sequence analysis of 16S rRNA gene sequences (Normand *et al.*, 1996) and of sequences of the 23S rRNA gene insertion (Hönerlage *et al.*, 1994). In addition to this basic classification of *Frankia* strains by host infection groups, phylogenetic analyses of both target genes identified a distinct, well supported cluster within the *Alnus* host infection group that consisted of those six strains that grew in leaf litter amended soil (Figure. 3.3). These results demonstrate the presence of a phylogenetically distinct lineage of frankiae within the *Alnus* host infection group that exhibit specific physiological properties.

The phylogenetic analyses demonstrated that *nifH* gene sequences were more discriminative between strains than those of the 23S rRNA gene insertion even though this insertion is meant to be hypervariable (Hönerlage *et al.*, 1994; Roller *et al.*, 1992). The basic tree topology, however, is congruent although slight differences with respect to position of individual strains exist depending on the target gene (Figure. 3.3). Both genes are therefore useful targets for molecular analyses of distinct *Frankia* clusters with different physiological properties. These studies could include analyses on the fate of non-growing cells in soil and the potential consequences for root nodule formation. In our study, strains that did not grow and could not be detected by *in situ* hybridization were still detectable after DAPI staining suggesting that they were inactive but still alive. Resuscitation of these cells by the addition of suitable C sources like root extracts (Krumholz *et al.*, 2003) or propionate (Caru *et al.*, 1997) and subsequent detection of growth could provide some insight on dormant stages of frankiae in soil, and help explain the detection of infective strains from environments not considered to be adequate

habitats for frankiae, such as river and lake sediments (Huss-Daniel *et al.*, 1997), partially decomposed wood (Li *et al.*, 1997), deep soil horizons (Nalin *et al.*, 1997), or soils stored long-term (i.e. up to 12 years) (Chaia *et al.*, 2007). Additional studies are definitely needed to analyze the potential activation of these cells in the rhizosphere of host or non-host plants and to assess the consequences of activation for nodule formation in competition with growing frankiae.

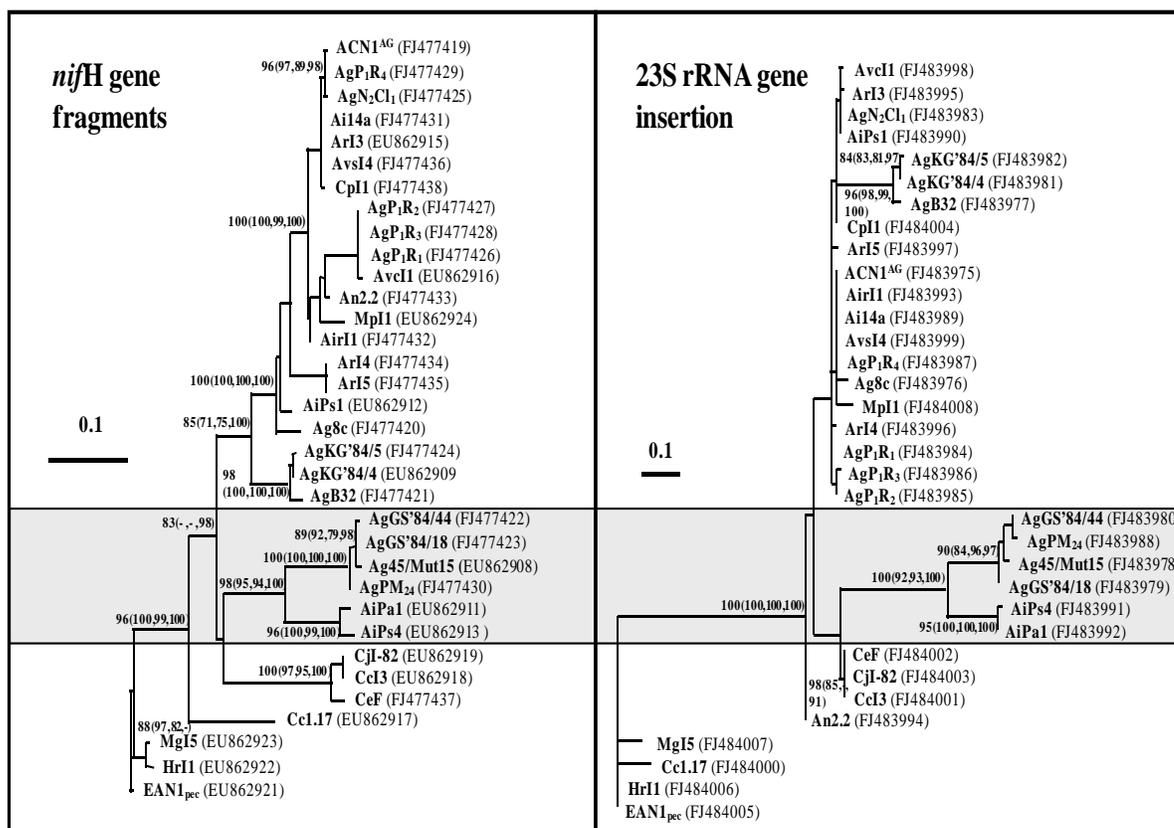


Figure 3.3: Phylogenetic grouping of *Frankia* strains on the basis of the *nifH* gene and actinomycete-specific insertion in Domain III of the 23S rRNA gene. Maximum likelihood-based tree generated using 552 bp of the *nifH* gene (left panel) or of 146 bp of the actinomycete-specific insertion in Domain III of the 23S rRNA gene (right panel) of the 34 *Frankia* strains tested. Numbers at nodes reflect bootstrap support measures (BS) and numbers in parentheses represent BS measures and posterior probabilities (PP) from neighbor joining, maximum parsimony, and Bayesian analyses, respectively. The outgroup was strain EAN1_{pec}. The shaded area marks strains of the *Alnus* host infection group found to grow in leaf litter amended soil.

Table 3.1: Details of 34 *Frankia* strains. *Frankia* strains used in the study.

Strain	Host plant	Origin	Reference
<i>Alnus</i> host infection group (sub-groups III and IV*)			
CpI1 (NRRL B-16285)	<i>Comptonia peregrina</i>	Petersham, MA, USA	(Callaham <i>et al.</i> , 1978)
ArI3 (NRRL B-16319)	<i>Alnus rubra</i>	Clackamas Co., OR, USA	(Berry & Torrey, 1979)
ArI4	<i>A. rubra</i>	Tacoma, WA, USA	(Baker <i>et al.</i> , 1981)
ArI5 (NRRL B-16403)	<i>A. rubra</i>	H.J. Andrews Exp. Forest, OR, USA	(Dillon & Baker, 1982)
AirI1 (NRRL B-16161)	<i>A. incana</i> ssp. <i>Rugosa</i>	Jefferson Heights, VT, USA	(Lechevalier <i>et al.</i> , 1983)
AvcI1 (ATCC 33255)	<i>A. viridis</i> ssp. <i>Crispa</i>	Atikokan, Ontario, Canada	(Baker <i>et al.</i> , 1979)
AvsI4 (NRRL B-16406)	<i>A. viridis</i> spp. <i>Sinuate</i>	Skykomish, WA, USA	(Baker, 1987)
ACN1 ^{AG}	<i>A. crisper</i>	Tadoussac, P.Q., Canada	(Normand & Lalonde, 1982)
AgP ₁ R ₁	<i>A. glutinosa</i>	Hoogmade, The Netherlands	(Burggraaf & Valstar, 1984b)
AgP ₁ R ₂	<i>A. glutinosa</i>	Hoogmade, The Netherlands	(Burggraaf & Valstar, 1984b)
AgP ₁ R ₃	<i>A. glutinosa</i>	Hoogmade, The Netherlands	(Burggraaf & Valstar, 1984b)
AgP ₁ R ₄	<i>A. glutinosa</i>	Hoogmade, The Netherlands	(Burggraaf & Valstar, 1984b)
AgKG'84/4	<i>A. glutinosa</i>	Krems-Goels, Germany	(Hahn <i>et al.</i> 1989)
AgKG'84/5	<i>A. glutinosa</i>	Krems-Goels, Germany	Unpublished
AgB32	<i>A. glutinosa</i>	Bad Bentheim, Germany	(Hahn <i>et al.</i> ,1989)
AiPs1	<i>A. incana</i>	Karttula, Finland	(Maunuksela <i>et al.</i> , 1999)
AiPa1	<i>A. incana</i>	Karttula, Finland	(Maunuksela <i>et al.</i> ,1999)
AiPs4	<i>A. incana</i>	Karttula, Finland	(Maunuksela <i>et al.</i> , 1999)
An2.2	<i>A. nitida</i>	Pakistan	(Chaudhary & Mirza, 1987)
AgGS'84/18	<i>A. glutinosa</i>	Grossensee, Germany	Unpublished
AgGS'84/44	<i>A. glutinosa</i>	Grossensee, Germany	Unpublished
Ag45/Mut15	<i>A. glutinosa</i>	Grossensee, Germany	(Hahn <i>et al.</i> , 1988)
Ai14a	<i>A. incana</i>	Rovaniemi, Finland	(Weber <i>et al.</i> , 1988)
AgN ₂ Cl ₁	<i>A. glutinosa</i>	Rijngest, The Netherlands	(Burggraaf ,1984a)
Ag8c	<i>A. glutinosa</i>	Kemijärvi, Finland	(Weber <i>et al.</i> , 1988)
AgPM ₂₄	<i>A. glutinosa</i>	The Netherlands	Unpublished
MpI1	<i>Myrica pensylvanica</i>	Nantucket, VT, USA	(Lechevalier & Lechevalier, 1979)
<i>Casuarina</i>-infective strains (sub-group II*)			
CcI3	<i>Casuarina cunninghamiana</i>	Tampa, FL, USA	(Zhang <i>et al.</i> , 1984)
CeF	<i>C. equisetifolia</i>	Miami, FL, USA	(Diem & Dommergues, 1983)
CjI-82	<i>C. junghuniana</i>	Bangkok, Thailand	(Diem <i>et al.</i> , 1983)
<i>Elaeagnus</i> host infection group (sub-group VI*)			
EAN1pec	<i>Elaeagnus angustifolia</i>	Ohio, USA	(Lalonde <i>et al.</i> , 1981)
MgI5 (NRRL B-16404)	<i>Myrica gale</i>	Tupper Lake, NY, USA	(Lechevalier, 1986)
HrI1 (NRRL B-16510)	<i>Hippophae rhamnoides</i>	Petersham, Ma, USA	(Hahn <i>et al.</i> ,1989)
Cc1.17	<i>Colletia cruciata</i>	The Netherlands	(Meesters <i>et al.</i> , 1985)

* according to Hönerlage *et al.* (1994)

Acknowledgements

The authors are indebted to the Texas State University-San Marcos Department of Biology and the National Science Foundation (GK-12 grant No. 0742306).

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CHAPTER IV

VARIATION IN *FRANKIA* POPULATIONS OF THE *ELAEAGNUS* HOST INFECTION GROUP IN NODULES OF SIX HOST PLANT SPECIES AFTER INOCULATION WITH SOIL

Abstract

The potential role of host plant species in the selection of symbiotic, nitrogen-fixing *Frankia* strains belonging to the *Elaeagnus* host infection group was assessed in bioassays with two *Morella*, three *Elaeagnus* and one *Shepherdia* species as capture plants, inoculated with soil slurries made with soil collected from a mixed pine/grassland area in central Wisconsin, USA. Comparative sequence analysis of *nifH* gene fragments amplified from homogenates of at least 20 individual lobes of root nodules harvested from capture plants of each species confirmed the more promiscuous character of *Morella cerifera* and *M. pensylvanica* that formed nodules with frankiae of the *Alnus* and the *Elaeagnus* host infection groups, while frankiae in nodules formed on *Elaeagnus umbellata*, *E. angustifolia*, *E. commutata* and *Shepherdia argentea* generally belonged to the *Elaeagnus* host infection group. Diversity of frankiae of the *Elaeagnus* host infection groups was larger in nodules on both *Morella* species than in nodules formed on the other plant

species. None of the plants, however, captured the entire diversity of nodule-forming frankiae. The distribution of clusters of *Frankia* populations and their abundance in nodules was unique for each of the plant species with only one cluster being ubiquitous and most abundant while the remaining clusters were only present in nodules of one (six clusters) or two (two clusters) host plant species. These results demonstrate large effects of the host plant species in the selection of *Frankia* strains from soil for potential nodule formation, and thus the significant effect of the choice of capture plant species in bioassays on diversity estimates in soil.

Introduction

Actinorhizal plants form root nodules in symbiosis with the nitrogen-fixing actinomycete *Frankia*, which enables them to grow on sites with restricted nitrogen availability (Akkermans *et al.*, 1992). They are therefore successful pioneer plants that are increasingly recognized in forestry and agroforestry for reforestation and reclamation of poor soils, but also for commercial use as nurse trees in mixed plantations with valuable tree species, for the production of fuelwood, and as a source of timber themselves (Dawson, 1983; Dawson, 1986; Gordon & Dawson, 1979; Gordon, 1983). The establishment and efficiency of the symbiosis between *Frankia* and actinorhizal plants such as *Alnus*, *Elaeagnus*, or *Casuarina* species is affected by environmental factors such as the soil pH (Crannell *et al.*, 1994; Griffiths & McCormick, 1984), the soil matrix potential (Dawson *et al.*, 1989; Schwintzer, 1985), and the availability of elements such as nitrogen (Kohls & Baker, 1989; Thomas & Berry, 1989) or phosphorus (Sanginga *et al.*, 1989; Yang, 1995), but ultimately constrained by the genotypes of both partners of this symbiosis (Hall *et al.*, 1979; Prat, 1989).

Effects of environmental conditions, plant species, and isolates of *Frankia* on the establishment of the symbiosis are relatively easy to assess under laboratory conditions and thus a considerable amount of information is available on isolates of *Frankia* and on their interaction with host plant species (see Benson & Silvester, (1993); Huss-Danell, (1997) for review). Quantitative analyses of specific *Frankia* populations originating from soil and their interaction with plants and site conditions, however, are methodologically extremely challenging due to problems encountered with isolation and identification of populations (Benson & Silvester, 1993; Schwintzer & Tjepkema, 1990), and consequently information on the occurrence and diversity of *Frankia* populations in soil is scarce. Until recently studies of *Frankia* populations in soil have been based solely on plant bioassays in which a quantification of the nodulation capacity on a specific host plant (expressed as nodulation units g^{-1} soil) is used to describe the infective *Frankia* population. This approach includes regression and most probable number (MPN) methods in which host plants inoculated with serial dilutions of *Frankia*-containing samples are statistically analyzed on the basis of nodule formation (Huss-Danell & Myrold, 1994).

The use of bioassays for quantitative assessments of *Frankia* populations, however, is hampered by its selectivity, since only nodule-forming populations can be detected. Questions on *Frankia* populations belonging to host infection groups other than the test plants (Baker, 1987), or on non-nodulating *Frankia* populations of the same host infection group (Hahn *et al.*, 1988) are therefore neglected. The correlation of the nodulation unit with cell numbers causes additional problems because a nodule can theoretically be induced by a single spore, a hyphal fragment, or a colony (Myrold *et al.*,

1994a). Other drawbacks of the bioassay include the failure to analyze non-competitive *Frankia* populations, including those present in low numbers, the inability to quantify competition for infection on the test plant between different *Frankia* populations in a sample, and intrageneric variation in host plants in compatibility with specific *Frankia* genotypes (Huss-Danell & Myrold, 1994; Maunuksela *et al.*, 2000).

While the selectivity of bioassays is well documented for frankiae belonging to a different host infection group than targeted by the test plant (Baker, 1987; Dobritsa *et al.*, 1990; Huang *et al.*, 1985), information on intrageneric variation in host plants in compatibility with specific *Frankia* populations is limited (Huss-Danell & Myrold, 1994; Maunuksela *et al.*, 2000). The purpose of the study was therefore to investigate potential variable compatibilities of different host plant species targeting *Frankia* populations of the same host infection group. Comparative sequence analyses of PCR-amplified *nifH* gene fragments was used to analyze uncultured *Frankia* populations in root nodules formed in plant bioassays after inoculation of slurries of the same soil on six plant species, i.e., two *Morella*, three *Elaeagnus* and one *Shepherdia* species, to cover potential effects between genera and between species. *Elaeagnus* and *Shepherdia* species were expected to only nodulate with *Frankia* populations of the *Elaeagnus* host infection group (Benson & Dawson, 2007), while *Morella* species were expected to be more promiscuous for frankiae, i.e., form nodules with frankiae belonging to the *Elaeagnus* host infection group, but also with those belonging to the *Alnus* host infection group (Baker, 1987; Dobritsa *et al.*, 1990; Huang *et al.*, 1985).

Materials and Methods

Soil collection: Soil was collected on September 7, 2006 from four, 10 x 10 m plots spaced every 300 m along a randomly-located 900 m transect within a disturbed mixed pine/grassland area at Fort McCoy, Wisconsin, USA (43°58'49"N, 90°43'36"W). Site and collection scheme were part of a larger project assessing the nodule-forming capacity of soils from different continents (Rieder and Paschke, unpublished). From each of the four plots, twenty 10-cm deep soil subsamples were collected, pooled, and homogenized, for a total of four pooled samples (referred to as A, B, C, and D). Between each plot, soil sampling equipment was sterilized to prevent cross-contamination, and the soils were kept cool and transported within 24 h to Colorado State University, where the soils were then refrigerated at 4°C until used in the experiment. The soil at the sampling site is classified as Tarr Sand, which formed from sandy residuum weathered from sandstone (SoilSurveyStaff, 2008). The only known native actinorhizal species in this habitat type is *Comptonia peregrina* (L.) J.M. Coult. (*Myricaceae*); however, neither this species nor other actinorhizal taxa were observed at the soil sampling area.

Plant propagation: Of the six actinorhizal plant species propagated, five species (*Morella cerifera* [L.] Small, *M. pensylvanica* [Mirb.] Kartesz, *Elaeagnus angustifolia* L., *E. umbellata* Thunb., and *E. commutata* Bernh. ex. Rydb.) were grown from seed, and one species (*Shepherdia argentea* [Pursh] Nutt.) was propagated from shoot cuttings. All seeds were surface sterilized in 30% H₂O₂ solution for 20 minutes, except those of *E. commutata* that were sterilized for only 3 minutes, and planted in autoclaved sand held in propagation flats. Shoot cuttings of *S. argentea* were surface sterilized by brief immersion in a 3% bleach solution, then dipped in Rootone[®] powder, and planted in

sterile sand held in propagation flats. Tools were sterilized between cuttings, and cuttings were housed on a mist bench until leaves emerged. Established cuttings and seed propagation flats were housed in the university greenhouses at Colorado State University on a 16:8 h light:dark schedule with supplemental lighting from 400 W sodium vapor lamps.

Once established, seedlings of each species ($n = 48$ individuals, except *S. argentea* [$n = 36$], *E. commutata* [$n = 27$], and *E. umbellata* [$n = 25$]) were transplanted to individual cone-tainer tubes (3.8 x 21 cm; Stuewe & Sons, Inc., Corvallis, OR) containing sterilized growth media (1:1:1 calcine clay/sand/vermiculite), and grown until a dense root mass filled the tube (approximately 3–6 months, depending on species). During the establishment phase, plants were provided with essential minerals and nutrients to sustain growth (Huss-Danell, 1978).

Inoculation: Two weeks prior to inoculation, the NH_4NO_3 was removed from the nutrient solution to encourage nodulation. Within a three-day period in December of 2006, plants in cone-tainers were inoculated with 5 ml of a soil slurry (20 cm^3 of soil in 100 ml of sterilized, distilled water), prepared for each of the four pooled samples from plots A, B, C, and D. Each of these samples was applied individually to the surface of the growth medium of a subset of plants in cone-tainers ($n = 5-11$ plants for each plot depending on the species). Un-inoculated sentinel plants ($n = 2-4$ per plant species) were maintained in the greenhouse beside the inoculated plants to monitor contamination from exogenous frankiae. Plants were maintained in the greenhouse with adequate water and N-free nutrient solutions. After about 6 months of growth, only two *S. argentea* plants had died, and the remaining plants of all species ($n = 230$) were harvested beginning in

June. Upon harvesting, a single nodule was collected from each individual that successfully nodulated (i.e., up to 11 nodules per plot and plant species). Nodules were preserved in 95% ethanol and stored at -20°C until further use.

Sample preparation: For each plant species, typically 5 nodules from each plot were selected for a total of 20 nodules (25 nodules for *M. pensylvanica*), and a single lobe from each nodule was used for DNA extraction. Lobes were washed with sterile water, and the periderm was removed with forceps while the preparation was viewed through a dissecting microscope. Remaining tissue was homogenized with a mortar and pestle in one ml of sterile water, and the homogenates transferred to an Eppendorf tube and centrifuged at 14,000 x g for one minute. The pellets were washed once with 0.1% sodium pyrophosphate in water (wt/vol), followed by two washes with sterile distilled water. Subsequently, the nodule pellets as well as pellets of pure cultures (approx. 50 mg) used for comparison, were re-suspended in 95 µl of distilled water, mixed with 5 µl of proteinase K solution (Promega, Madison, WI, 30 U mg⁻¹, 10 mg ml⁻¹ in water) and incubated at 37°C for 20 minutes. Afterwards, 0.5 µl of 10% SDS solution was added and the mixtures incubated at 37°C for another 3 hours which was followed by a final incubation at 80°C for 20 minutes. From these lysates, 2 µl were used as template in subsequent PCR-based analyses.

PCR amplification: *NifH* gene fragments (606 bp) were amplified using primers *nifHf1* (5' GGC AAG TCC ACC ACC CAG C) and *nifHr* (5' CTC GAT GAC CGT CAT CCG GC) (Normand *et al.*, 1988) in a reaction volume of 50 µl, containing 1 µl of a 10 mM dNTP mix, 0.5 µl each primer (0.4 µM), 8.2 µl BSA (30 µg ml⁻¹), 5 µl of 10 x PCR buffer with 15 mM MgCl₂, 2 µl root nodule or pure culture lysate, and 0.2 µl *Taq* DNA

polymerase ($5 \text{ U } \mu\text{l}^{-1}$; Gene Script, Piscataway, NJ) that was added after an initial incubation at 96°C for 10 minutes. The addition of *Taq* polymerase was followed by 35 rounds of temperature cycling (96°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 second) and a final 7 minute incubation at 72°C . Sub-samples of the reactions ($5 \mu\text{l}$) were checked for amplification products by gel electrophoresis (1% agarose in TAE buffer, wt/vol) after staining with ethidium bromide ($0.5 \mu\text{g ml}^{-1}$) (Sambrook *et al.*, 1989).

Rep-PCR patterns were generated for selected samples using primer BoxA1R ($5'$ CTA CGG CAA GGC GAC GCT GAC G) (Versalovic *et al.*, 1998). PCR was performed in a total volume of $25 \mu\text{l}$ containing $4 \mu\text{l}$ of 5 x Gitschier buffer (83 mM $(\text{NH}_4)_2\text{SO}_4$, 33.5 mM MgCl_2 , 335 mM Tris/HCl, pH 8.8, $33.5 \mu\text{M}$ EDTA, 150 mM β -mercaptoethanol), $1.25 \mu\text{l}$ of dNTPs (100 mM each, mixed 1:1:1:1), $2.5 \mu\text{l}$ di-methyl-sulfoxide (DMSO), $0.2 \mu\text{l}$ bovine serum albumin (BSA, 20 mg ml^{-1}), $1.3 \mu\text{l}$ of primer ($300 \text{ ng } \mu\text{l}^{-1}$), $0.4 \mu\text{l}$ of *Taq* polymerase ($5 \text{ U } \mu\text{l}^{-1}$) (Rademaker & de Bruijn, 1997), and $2 \mu\text{l}$ of lysate (Dombek *et al.*, 2000). After an initial incubation at 96°C for 10 minutes and the subsequent addition of *Taq* polymerase, 30 rounds of temperature cycling were performed in a PTC-200 Thermocycler with denaturation at 94°C for 3 seconds and subsequent 92°C for 30 seconds, primer annealing at 50°C for 1 minute, and elongation at 65°C for 8 minutes, then a final elongation incubation at 65°C for 8 minutes (Dombek *et al.*, 2000; Rademaker & de Bruijn, 1997). Profiles were analyzed by gel electrophoresis on 2% agarose gels in TAE buffer after staining with ethidium bromide ($0.5 \mu\text{g ml}^{-1}$).

Sequence analyses: Amplified *nifH* gene fragments were purified using the Ultra Clean 15 DNA Purification Kit (MoBio, Carlsbad, CA), and sequenced using the CEQ 8800 Quickstart Kit according to the manufacturer's instructions (Beckman Coulter, Fullerton, CA) with the addition of 5% DMSO to the reaction mix. The sequencing reaction consisted of an initial incubation at 76°C for 5 minutes followed by 76°C for 5 minutes during which primer and master mix were added, a subsequent incubation at 94°C for 2 minutes, and 35 cycles of temperature cycling (94°C for 30 seconds, 50°C for 30 seconds and 60°C for 4 minutes) and a final extension at 60°C for 10 minutes (Kukanskis *et al.*, 1999). Sequences were analyzed on a CEQ 8800 sequencer (Beckman Coulter) and deposited at Genbank under accession numbers EU862907 to EU863053.

Phylogenetic analyses: Amplified *nifH* gene fragments obtained from uncultured frankiae from root nodules of all plants analyzed and those of 18 pure cultures of *Frankia* were trimmed to be 543 bp long and aligned using Sequencher 4.2.2 (Gene Codes Corp., Ann Arbor, MI), CLUSTAL X and MacClade 4.05 (Maddison & Maddison, 1999; Thompson *et al.*, 1997) and analyzed using maximum parsimony (MP), neighbor joining (NJ), Bayesian and maximum likelihood (ML) methods.

MP methods began by using MacClade 4.05 to chart the rate of mutation by codon position. Third position changes were downweighted and thus first position changes upweighted to better delineate meaningful changes, i.e. in protein structure, between these sequences. MP analyses of these weighted datasets were completed in PAUP*4.0b10 and included 10,000 heuristic random addition replicates, TBR, and no mul trees (Swofford, 2002). Confidence in the topology for this MP tree was gauged using bootstrap re-sampling methods (BS) in PAUP* and included 10,000 replications

and a full heuristic search (Felsenstein, 1985). Only those BS values of at least 70% demonstrate good support measures and thus were retained (Hillis & Bull, 1993).

The dataset was analyzed using NJ methods in PAUP*. Modeltest version 3.7 using the Akaike Information Criterion determined that the GTR+I+G model of sequence evolution fit this dataset best (Posada & Crandell, 1998). Specific values for the gamma shape parameter and proportion of invariant sites provided by Modeltest were entered under the distance settings for the NJ GTR model in PAUP*. The BS test in PAUP* included 10,000 replications and a neighbor joining search.

Bayesian analysis for the *Frankia* dataset was completed using MRBAYES version 3.0 and included Metropolis-coupled Markov chain Monte Carlo (MCMCMC) sampling, a GTR+I+G model estimated during the run, 5 million generations, and sampling every 1000 trees (Huelsenbeck & Ronquist, 2001). A 95% majority rule consensus tree for the Bayesian output of posterior probabilities (PP) was created in PAUP* with the first 30 trees removed as burn-in (Huelsenbeck *et al.*, 2002; Swofford, 2002).

Maximum likelihood analysis was completed using the RAxML-VI-HPC program (Stamatakis, 2006b) on the computer cluster of the 'CyberInfrastructure for Phylogenetic REsearch' project (CIPRES, www.phylo.org) from the online servers at the San Diego Supercomputing Center. The RAxML program is designed for fast processing of large datasets. Settings included GTR+CAT approximation for rate heterogeneity (Stamatakis, 2006a), invariant sites, empirical base frequencies and required bootstrap replicates estimated during the run.

Frankia strains and uncultured populations from nodules were assigned to specific clusters based on known genomic groupings and data from distance matrices of the

datasets. *Frankia* strains AvcI1, An2.1, MpI1, and ArI3 belonging to the *Alnus* host infection group included in these analyses have been shown to belong to the same genomic group (Akimov & Dobritsa, 1992; An *et al.*, 1985; Fernandez *et al.*, 1989). Cluster assignments were based on sequence similarity with values higher than 97% which is the lowest similarity value that would still group all these pure cultures of the same genomic group into one cluster. However, *Frankia* strains Ea1.12 and EAN1pec of the *Elaeagnus* host infection group have been shown to be in different genomic groups (Fernandez *et al.*, 1989) and are 98.8% similar in this portion of the *nifH* gene. Thus, the diversity of *Frankia* revealed by these clustering assignments is conservative and underestimates the total diversity present. Cluster assignments were considered supported when BS values were higher than 70% (Hillis & Bull, 1993) and PP values higher than 95% (Huelsenbeck *et al.*, 2002), in at least three of the four phylogenetic methods employed. TreeView (Win16) was used to display the treefiles (Page, 1996) and MEGA version 4 was used to collapse branches (Tamura *et al.*, 2007) (Figure. 4.1). For a more detailed presentation of the phylogenetic position of uncultured *Frankia* populations in root nodules of specific host plant species, the dataset was split into six datasets containing sequences of pure cultures and of uncultured frankiae for each plant species, and each dataset was reanalyzed (Figure. 4.2, 4.3, 4.4).

Results and discussion

NifH gene fragments were obtained from crude lysates of all nodule lobe homogenates tested, i.e., of 25 samples from *M. pensylvanica* and 20 samples each of *M. cerifera*, *E. angustifolia*, *E. umbellata*, *E. commutata* and *S. argentea*. Phylogenetic analyses based on *nifH* gene sequence analyses revealed similar tree topologies and grouping of frankiae

from pure cultures and root nodules for each method employed (data not shown), and no variation in populations among plots A, B, C, or D because sequences that clustered together were generally obtained from several, even though not necessarily all plots (i.e., 3 out of 4 plots). Thus, all sequences from nodules were pooled and not differentiated by plot in the final presentation. Based upon the definition of clusters having sequence similarity with values higher than 97%, overall 9 different clusters of frankiae were identified in nodules on the six plant species, i.e., two clusters belonging to the *Alnus* host infection group (A I and A II), and seven clusters of the *Elaeagnus* host infection group (E I to E VII) (Figure. 4.1). These results demonstrate a large diversity of *Frankia* populations present in soil lacking known host plants and being able to form root nodules. This observation is consistent with findings on diversity of frankiae on field-collected nodules (Clawson *et al.*, 1998; Clawson & Benson, 1999).

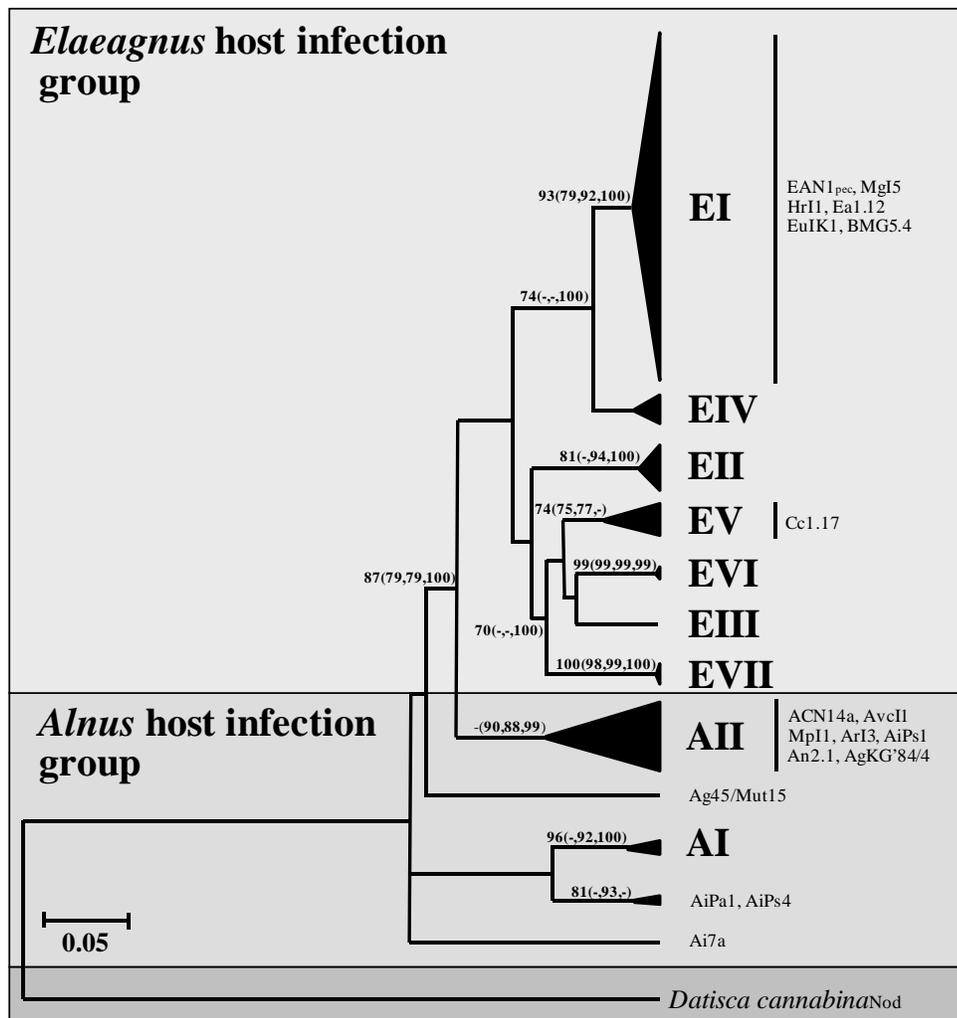


Figure 4.1: Comparative sequence analyses of uncultured *Frankia* populations in the root nodules of six plant species on the basis of the *nifH* gene. Maximum likelihood-based tree showing an overall analysis of 19 pure cultures of *Frankia* and 129 sequences representing uncultured *Frankia* populations in root nodules based on comparative sequence analysis of 543 bp *nifH* gene fragments. The light shaded areas depict sequence assignments to the *Elaeagnus* host infection group with cluster acronyms E I to EVII, while the darker shaded areas represent sequences assigned to the *Alnus* host infection group with cluster acronyms A I and AII. Clusters represent frankiae with less than 3% sequence variation. Branches within clusters were collapsed to show the overall relationships of the clusters to one another. Pure cultures present in each cluster are listed on the right. Numbers reflect maximum likelihood bootstrap support measures (BS) and numbers in parentheses represent BS measures and posterior probabilities (PP) from maximum parsimony, neighbor joining and Bayesian analyses, respectively. The outgroup was a sequence of an uncultured *Frankia* population from nodules of *Datisca cannabina* (AY768412).

Frankia populations found in nodules on both *M. cerifera* and *M. pennsylvanica* included several populations belonging to the *Alnus* host infection group (Figure. 4.2) which confirms the assumption that *Morella* species are promiscuous and can form root nodules with frankiae belonging to different host infection groups (Clawson & Benson, 1999). Except for one nodule on *E. angustifolia*, *Frankia* populations belonging to the *Alnus* host infection group were absent in nodules obtained on the *Elaeagnus* and *Shepherdia* species (Figure. 4.3, 4.4), which is in agreement with previous studies (Clawson *et al.*, 1998; Dobritsa *et al.*, 1990; Huang *et al.*, 1985; Huguet *et al.*, 2001).

The assignment of the *nifH* gene fragment sequence obtained from the nodule of *E. angustifolia* to the *Alnus* host infection group (cluster A II) is supported by high BS and PP values (-,90,88,99) in the presence of all sequences (Figure. 4.1). *Frankia* strains that were able to cross boundaries between *Alnus* and *Elaeagnus* host infection groups have been described before (Bosco *et al.*, 1992; Margheri *et al.*, 1989); however, these strains were retrieved from nodules of an *Elaeagnus* species and thus were identified as belonging to the *Elaeagnus* host infection group producing nodules on alders, and thus not to the *Alnus* host infection group. Since only sequence information and no isolate of this population is available, any further interpretation of this finding remains highly speculative. The detection of this sequence, for example, might just reflect a methodological artifact due to the detection of a strain present on the surface and in tissue of this nodule detected as a consequence of incomplete surface sterilization or periderm removal (Simonet *et al.*, 1999). *NifH* genes have also been found on plasmids in some frankiae (Simonet *et al.*, 1986) and thus horizontal gene transfer among distantly related

Frankia might be an alternative direction for speculations on the origin of this sequence (Hirsch *et al.*, 1995; Manjula & Rakesh, 1990).

In addition to the presence of frankiae belonging to the *Alnus* host infection group, *Frankia* populations found in nodules on both *M. cerifera* and *M. pensylvanica* belonging to the *Elaeagnus* host infection group were more diverse than those in nodules on *Elaeagnus* and *Shepherdia* species. *Frankia* populations in both *Morella* species differed from each other, with populations of the *Alnus* host infection groups being entirely different, and those of the *Elaeagnus* host infection groups being represented by one unique cluster each (E III in *M. cerifera*, and E IV in *M. pensylvanica*) in addition to two shared clusters (E I and E II) (Figure. 4.2). Abundance of sequences assigned to these clusters varied between plant species. In nodules of *M. cerifera*, frankiae of the *Alnus* and *Elaeagnus* host infection groups were represented by 15% (A I), 55% (E I), 25% (E II), and 5% (E III) of the sequences, respectively, while those in nodules of *M. pensylvanica* were represented by 36% (A II), 4% (E I), 24% (E II), and 36% (E IV) of the sequences (Figure. 4.2).

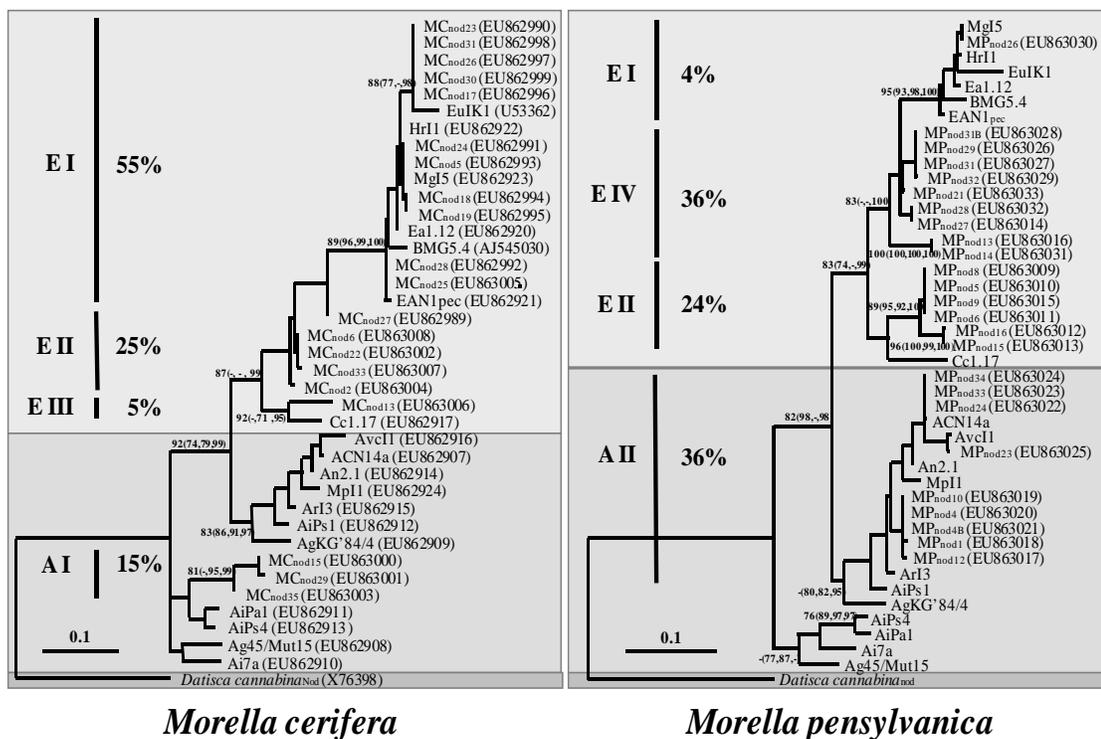


Figure 4.2: Comparative sequence analyses of uncultured *Frankia* populations in the root nodules of *Morella cerifera* and *Morella pensylvanica*. Maximum likelihood-based tree showing the phylogenetic position of uncultured *Frankia* populations in root nodules of *Morella cerifera* (MCnod) (left panel) and *Morella pensylvanica* (MPnod) (right panel) based on comparative sequence analysis of *nifH* gene fragments. The light shaded areas depict sequence assignments to the *Elaeagnus* host infection group with cluster acronyms E I to E III for *M. cerifera*, and acronyms E I to E IV for *M. pensylvanica*, while the darker shaded areas represent sequences assigned to the *Alnus* host infection group with cluster acronyms A I, and AII for *M. cerifera* and *M. pensylvanica*, respectively. Numbers reflect maximum likelihood bootstrap support measures (BS) and numbers in parentheses represent BS measures and posterior probabilities (PP) from maximum parsimony, neighbor joining and Bayesian analyses, respectively. The outgroup was a sequence of an uncultured *Frankia* population from nodules of *Datisca cannabina* (AY768412).

Diversity of frankiae in nodules on *Shepherdia argentea* and the three *Elaeagnus* species differed between these plant species and, with the exception of *E. commutata*, was usually lower with 1 or 2 different clusters than that detected on both *Morella* species

(Figure. 4.3, 4.4). *Frankia* populations in nodules of *E. umbellata*, for example, were all assigned to cluster E I. Similarly small diversity was obtained for *S. argentea* where 85% of the nodules harbored frankiae of cluster E I, while the remaining 15% were represented by cluster E V (Figure. 4.3). These results were similar to those obtained for *E. angustifolia* (80% E I and 15% E VI), while nodules on *E. commutata* harbored a third cluster (25% E VII) in addition to clusters E I (55%) and E V (20%) (Figure. 4.4). Several of the clusters of frankiae were unique for the plant species, i.e., E III for *M. cerifera* (5% of the nodules analyzed) and E IV for *M. pensylvanica* (36%). Other unique clusters were E VI for *E. angustifolia* (15%) and E VII for *E. commutata* (25%) (Figure. 4.4).

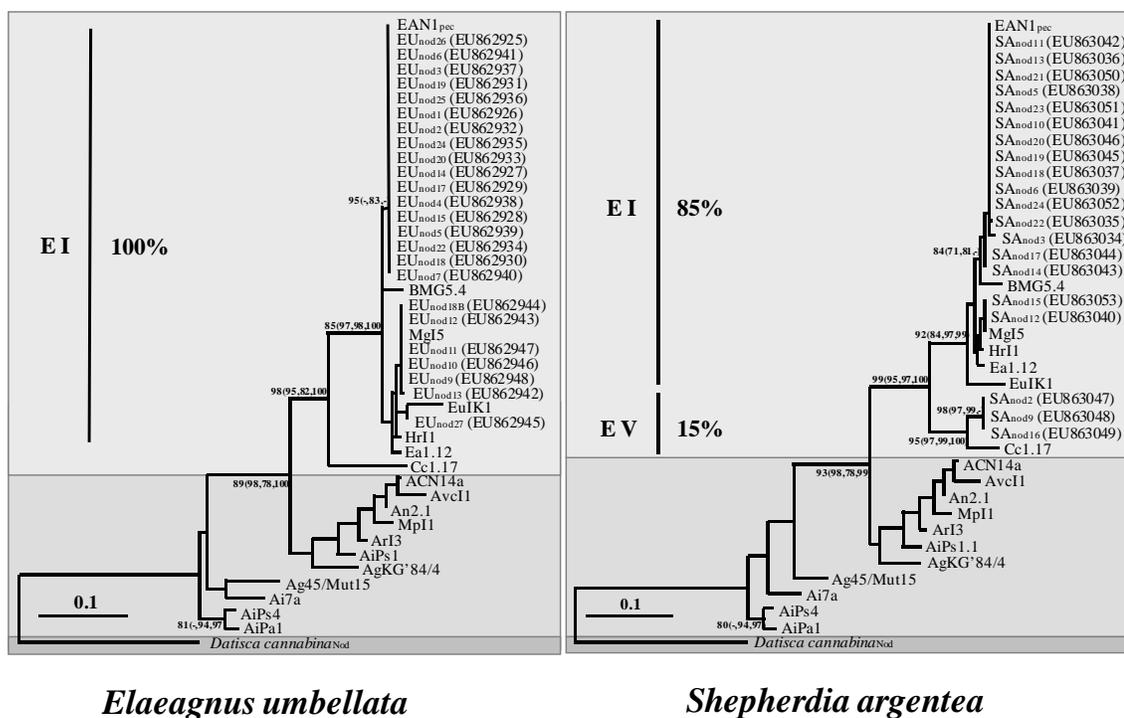


Figure 4.3: Comparative sequence analyses of uncultured *Frankia* populations in the root nodules of *Elaeagnus umbellata* and *Shepherdia argentea*. Maximum likelihood-based tree showing the phylogenetic position of uncultured *Frankia* populations in root nodules of *Elaeagnus umbellata* (EUnod) (left panel) and *Shepherdia argentea* (SAnod) (right panel) based on comparative sequence analysis of *nifH* gene fragments. The light shaded areas depict sequence assignments to the *Elaeagnus* host infection group with cluster acronym E I for *E. umbellata*, and acronyms E I and E V for *S. argentea*, while the darker shaded areas represent sequences assigned to the *Alnus* host infection group with no sequences detected for either *E. umbellata* or *S. argentea*, respectively. Numbers reflect maximum likelihood bootstrap support measures (BS) and numbers in parentheses represent BS measures and posterior probabilities (PP) from maximum parsimony, neighbor joining and Bayesian analyses, respectively. The outgroup was a sequence of an uncultured *Frankia* population from nodules of *Datisca cannabina* (AY768412).

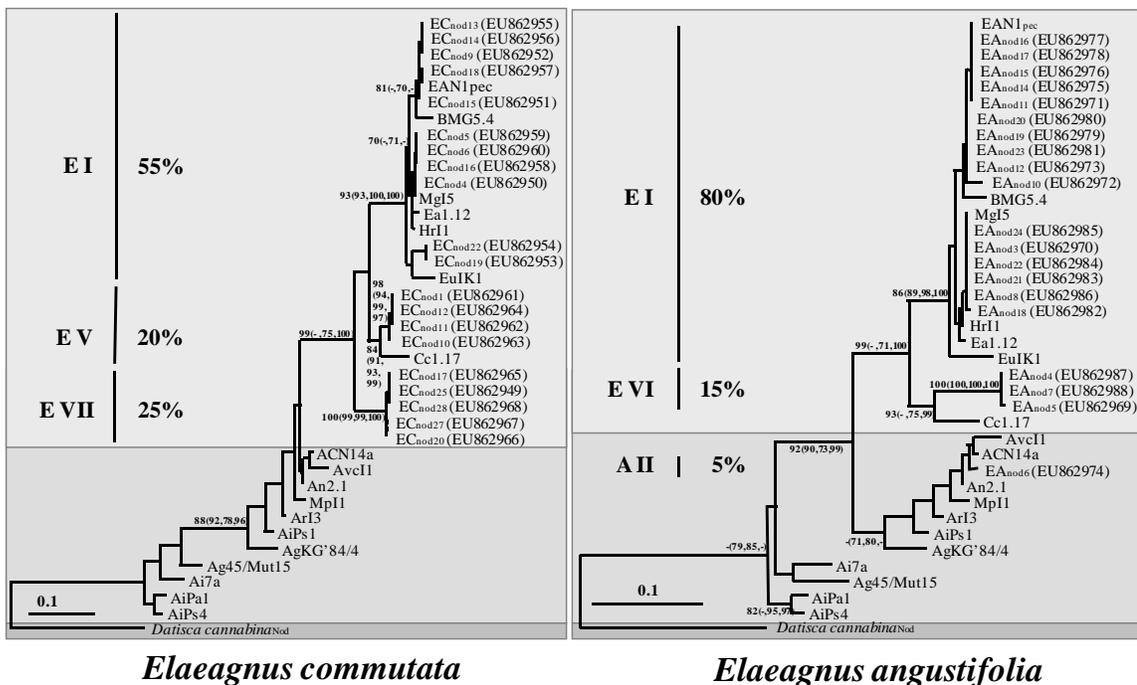


Figure 4.4: Comparative sequence analyses of uncultured *Frankia* populations in the root nodules of *Elaeagnus commutata* and *Elaeagnus angustifolia*. Maximum likelihood-based tree showing the phylogenetic position of uncultured *Frankia* populations in root nodules of *Elaeagnus commutata* (EC_{nod}) (left panel) and *Elaeagnus angustifolia* (EA_{nod}) (right panel) based on comparative sequence analysis of *nifH* gene fragments. The light shaded areas depict sequence assignments to the *Elaeagnus* host infection group with cluster acronyms E I, E V and E VII for *E. commutata*, and acronyms E I and E VI for *E. angustifolia*, while the darker shaded areas represent sequences assigned to the *Alnus* host infection group with no sequences detected for *E. commutata*, but one for *E. angustifolia* (A II). Numbers reflect maximum likelihood bootstrap support measures (BS) and numbers in parentheses represent BS measures and posterior probabilities (PP) from maximum parsimony, neighbor joining and Bayesian analyses, respectively. The outgroup was a sequence of an uncultured *Frankia* population from nodules of *Datisca cannabina* (AY768412).

Cluster E I represented the only ubiquitous populations that were also usually found in the majority of nodules. Cluster E I also harbored the majority of cultured relatives for which sequences were available (e.g., HrI1 (*Hippophaë rhamnoides*, USA), BMG5.4 (*E. angustifolia*, Tunisia), EAN1pec (*E. angustifolia*, USA), MgI5 (*Myrica gale*, USA) or

Ea1.12 (*E. angustifolia*, France)). This, together with the widespread origin of these strains with respect to host plant species and location indicates a certain prominence of members of this cluster in nature. Except for cluster E V that contained strain Cc1.17 (*Colletia cruciata*, The Netherlands) and was present in a small number of nodules of *S. argentea* (15%) and *E. commutata* (20%) only (Figure. 4.3, 4.4), none of the remaining clusters of the *Elaeagnus* host infection group was represented by a cultured relative which, however, might be due to the small dataset of sequences available from cultured strains of this host infection group.

These data indicate the presence of a diverse community of nodule-forming frankiae in this particular soil. Despite a cut-off level at 97% sequence similarity (i.e., up to 16 nucleotides different within the same cluster), 9 clusters of frankiae were identified. Thus, if single nucleotide differences in sequences of *nifH* gene fragments were considered discriminatory, the diversity of frankiae would further increase. Even identical sequences can represent different strains if analyzed with fingerprinting methods like rep-PCR that provide a better resolution on the strain level (Murry *et al.*, 1995; Murry *et al.*, 1997; Versalovic *et al.*, 1994). Fingerprinting of populations in nodules of *S. argentea* by rep-PCR, for example, revealed at least three distinct profiles in a sub-cluster of cluster E I (Figure. 4.5) characterized by sequence identity or very high sequence similarity (Figure. 4.3). Strain EAN1pec that had an identical sequence to *Frankia* populations in 13 root nodules on this plant species displayed a fourth distinct profile (data not shown). Thus, if single nucleotide differences in *nifH* gene fragments would have been considered discriminatory, and rep-PCR profiles been consistently added to sequence analysis data in

our study, the overall diversity of *Frankia* strains in soil would have exceeded the current assignment of 9 clusters.

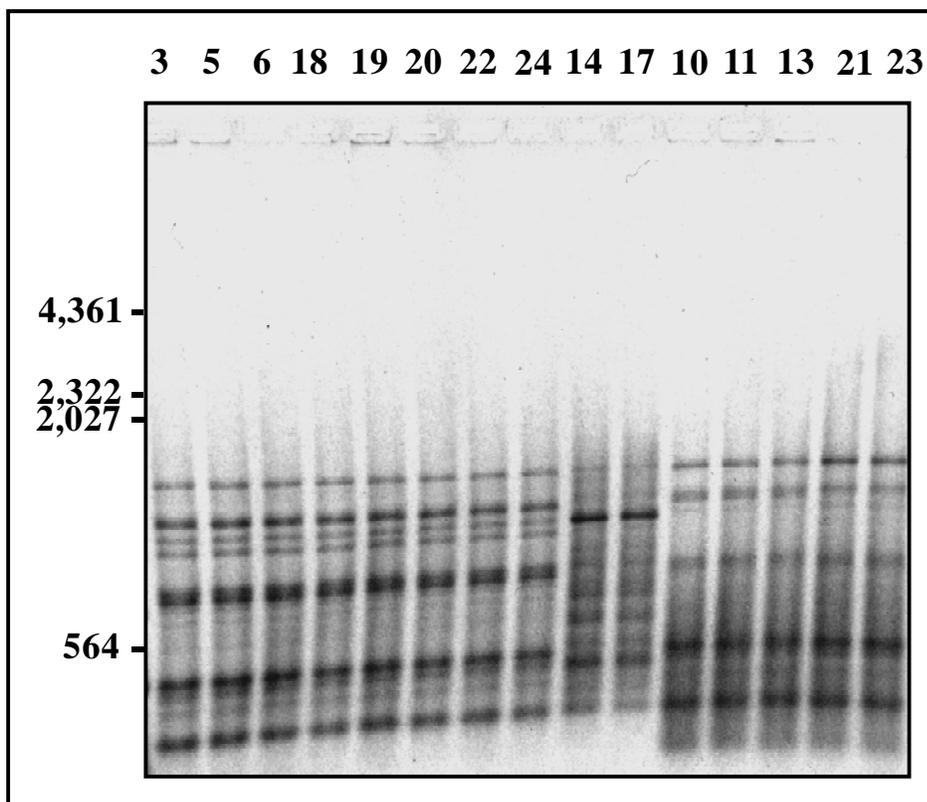


Figure 4.5: Rep-PCR profiles of uncultured *Frankia* populations in root nodules of *Shepherdia argentea*. Rep-PCR profiles of uncultured *Frankia* populations in root nodules (numbers 3 – 23, corresponding to SAnod3 to SAnod23 in a subgroup of cluster E I, Figure. 3) from *Shepherdia argentea* that show identical or highly similar sequences of *nifH* gene fragments (Figure. 3). Fragment sizes on the left represent those of a Lambda HindIII size marker.

Since each test plant species only formed nodules with frankiae of between 1 to 3 clusters for each host infection group, none of the plants captured the entire diversity of frankiae retrieved from this soil even on the current cluster assignment. Also, the distribution of clusters of *Frankia* populations and their abundance in nodules was unique for each of the plant species with several clusters only present in nodules of one (A I, E III, E IV, E VI, E VII) or two (A II, E II, E V) host plant species. These results demonstrate large

effects of the host plant species on the selection of *Frankia* populations for nodule formation. Results of plant bioassays as described here, however, will not necessarily reflect results obtained from field studies where environmental conditions might obscure plant species effects on nodule formation. This was demonstrated for *Frankia* populations in nodules of *Myrica gale* that was described as being promiscuous based on bioassays (Torrey, 1990); however, populations in field-collected nodules showed much less diversity than anticipated or compared to other plant species like *M. pennsylvanica* (Clawson & Benson, 1999; Huguet *et al.*, 2001; Huguet *et al.*, 2004).

Despite the relatively small sampling size (20 to 25 nodule lobes), rarefaction analyses show that the entire diversity of *Frankia* populations in root nodules has likely been captured for most of the plants, except for *M. cerifera* and *E. angustifolia* (data not shown). The limited number of clusters generally represented by several identical or very similar sequences obtained from different nodules and plants suggests that these plant species have an effect on nodulation by different *Frankia* populations of the same host infection group. Since *Frankia* populations in nodules differ with respect to diversity and abundance, the choice of the capture plant species in bioassays will have a significant effect on diversity estimates in soil. Since nodules of all plant species were occupied by one major population, a much larger sampling size of nodules might be necessary to retrieve information on the presence and abundance of low-frequency populations or account for intra-specific variation of diversity on individual test plants (Chávez & Carú, 2006).

Acknowledgements

The authors thank Dr. Laura G. Perry and the Restoration Ecology Laboratory Crew (Liza Bodistow, Bryan Brown, Jeremy Buss, Chris Herron, Lilly Hines, Tim Hoelzle, Ben Hoffman, Erin Klamper, Katie Legg, Mandy Roesch, Travis Talbot, Hannah Varani, Will Vieth) for development of protocols, propagation of plant species, maintenance of the experiment, and harvesting plants. The authors are indebted to the Texas State University-San Marcos Department of Biology, the National Science Foundation (GK-12 grant No. 0742306) and the Higher Education Commission of Pakistan for financial support.

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CHAPTER V

DIVERSITY OF FRANKIAE IN SOILS FROM FIVE CONTINENTS

Abstract

Clone libraries of *nifH* gene fragments specific for the nitrogen-fixing actinomycete *Frankia* were generated from six soils obtained from five continents using a nested PCR. Comparative sequence analyses of all libraries (n=247 clones) using 96 to 97% similarity thresholds revealed the presence of three and four clusters of frankiae representing the *Elaeagnus* and the *Alnus* host infection groups, respectively. Diversity of frankiae was represented by fewer clusters (i.e., up to four in total) within individual libraries, with one cluster generally harboring the vast majority of sequences. Meta-analysis including sequences previously published for cultures (n=48) and for uncultured frankiae in root nodules of *Morella pensylvanica* formed in bioassays with the respective soils (n=121) revealed a higher overall diversity with four and six clusters of frankiae representing the *Elaeagnus* and the *Alnus* host infection groups, respectively, and displayed large differences in cluster assignments between sequences retrieved from clone libraries and those obtained from nodules, with assignments to the same cluster only rarely encountered

for individual soils. These results demonstrate large differences between detectable *Frankia* populations in soil and those in root nodules indicating the inadequacy of bioassays for the analysis of frankiae in soil and the role of plants in the selection of frankiae from soil for root nodule formation.

Key words: clone libraries - *Frankia* - *nifH* - nitrogenase – root nodules

Introduction

Studies on the diversity of *Frankia* sp., the nitrogen-fixing symbiotic partner of actinorhizal plants generally focus on populations in root nodules formed on host plants like *Alnus* or *Elaeagnus* species (Benson & Dawson, 2007; Hahn *et al.*, 1999). Root nodules represent a natural locale of enrichment of usually one *Frankia* population, and thus allow researchers to retrieve information on this population relatively easily using a variety of molecular tools that include PCR-assisted sequence analyses or *in situ* hybridization (Hahn *et al.*, 1997; Welsh *et al.*, 2009)], but also fingerprinting techniques such as rep-PCR or PCR-RFLPs that usually require pure cultures (McEwan *et al.*, 1994; Murry *et al.*, 1997). Consequently, *Frankia* populations in root nodules are well studied and a considerable amount of information is available on the *Frankia* and their interaction with host plants (Benson & Silvester, 1993; Huss-Danell, 1997). Information on frankiae in soil, the second ecological niche that frankiae inhabit, however, is much more limited. Soil represents a highly heterogeneous environment that supports a large microbial community (more than 10^9 cells g^{-1} soil) (Chatzinotas *et al.*, 1998; Zarda *et al.*, 1997) at a tremendous diversity (Dunbar *et al.*, 2002; Gans *et al.*, 2005; Torsvik *et al.*,

1990; Torsvik *et al.*, 1996). Frankiae are present in small numbers in soils (approximately 10^4 - 10^5 cells g^{-1} soil) (Hahn *et al.*, 1990; Myrold *et al.*, 1994a), which is close to the detection limit of commonly used PCR-based analysis tools.

Except for a few investigations (Myrold *et al.*, 1990; Myrold *et al.*, 1994a; Myrold & Huss-Danell, 1994b), studies on frankiae in soil have frequently relied on plant bioassays in which nodule-forming frankiae were identified on roots of a specific host plant growing in soil or after inoculation with a soil slurry (Maunuksela *et al.*, 1999; Maunuksela *et al.*, 2000; Nickel *et al.*, 1999; Welsh *et al.*, 2009; Zepp *et al.*, 1997). Bioassays using *Morella pensylvanica* as capture plants, for example, have demonstrated large differences in nodule-forming frankiae in five soils from a broad geographic range, i.e., from sites in five continents (Africa, Europe, Asia, North America and South America), but a low diversity of nodule-forming *Frankia* populations within any of these soils (Welsh *et al.*, 2009). This approach, however, is hampered by the potential of the host plant to select specific *Frankia* strains for root nodule formation as shown in bioassays with two *Morella*, three *Elaeagnus* and one *Shepherdia* species as capture plants inoculated with the same soil slurry (Mirza *et al.*, 2009). This study demonstrated that none of the plants captured the entire diversity of nodule-forming frankiae and that the distribution of *Frankia* populations and their abundance in nodules was unique for each of the plant species (Mirza *et al.*, 2009). As a consequence, the diversity of *Frankia* populations in nodules retrieved by plant bioassays might therefore reflect preferences of the host plant rather than describe the diversity of frankiae in the soil analyzed.

The aim of this study was to determine the diversity of frankiae directly in those six soils that had been analyzed using bioassays with *M. pensylvanica* in previous studies (Mirza *et al.*, 2009; Welsh *et al.*, 2009). As in these previous studies, diversity assessments of frankiae were based on comparative sequence analyses of *nifH* gene fragments. Due to the heterogeneity of the microbial community and the low abundance of frankiae in these soils, however, a nested PCR approach using *Frankia*-specific primers was necessary to provide enough product to generate clone libraries. Clone libraries were analyzed by different phylogenetic methods and sequences compared to those obtained from uncultured frankiae in root nodules of *M. pensylvanica* (Welsh *et al.*, 2009).

Materials and methods

Sample preparation: Soil samples were collected in October 2006 from four plots at sites located in 5 continents as described previously (Mirza *et al.*, 2009; Welsh *et al.*, 2009). Sites included one each in Africa (Rwanda, S 1°56'45.76", E 30°03'13.40"), Europe (Hungary, N 46°52'24.52", E 19°23'19.64"), Asia (Japan, N 38°43'8.88", E 139°51'29.00"), South America (Peru, S 6°57'25", W 78°22'48"), and two in North America (Alaska, USA, N 61°10'07.19", W 149°45'39.43", and Wisconsin, USA, 43°58'49"N, 90°43'36"W). Soils were stored at Colorado State University at 4°C until used for DNA extraction.

For DNA extraction, 0.5 g of soil from each of the four sub plots from a single site was mixed and a 1-gram-sample from each site used for DNA extraction after bead beating for two minutes in a Mini-Bead-Beater-8 (BioSpec Products, Inc, Bartlesville, OK) (Hönerlage *et al.*, 1995). Nucleic acids released were purified by sequential phenol, phenol/chloroform and chloroform extraction (Sambrook *et al.*, 1989), which was

followed by precipitation with two volumes of 2.5 M NaCl/20% PEG 8000 (Widmer *et al.*, 1999). After an additional extraction with phenol/chloroform and subsequent mixing of the supernatant with one volume of isopropanol, nucleic acids were precipitated by centrifugation at 13,000 rpm for 10 minutes, washed with 70% ethanol, air-dried and finally re-suspended in 20 μ l of sterile distilled water.

PCR amplification: Nucleic acids in 2 μ l of this solution were used as template to amplify *nifH* gene fragments (606 bp) using primers *nifHf1* (5' GGC AAG TCC ACC ACC CAG C) and *nifHr* (5' CTC GAT GAC CGT CAT CCG GC) in a reaction volume of 50 μ l, containing 1 μ l of a 10 mM dNTP mix, 0.5 μ l each primer (0.4 μ M), 8.2 μ l BSA (30 μ g ml⁻¹), 5 μ l of 10 x PCR buffer with 15 mM MgCl₂, 2 μ l of template DNA, and 0.2 μ l *Taq* DNA polymerase (5 U μ l⁻¹; Gene Script, Piscataway, NJ) that was added after an initial incubation at 96°C for 10 minutes. The addition of *Taq* polymerase was followed by 35 rounds of temperature cycling (96°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 second) and a final 7-minute incubation at 72°C.

The product of this PCR reaction that was generally invisible when analyzed by gel electrophoresis was purified using the Ultra Clean 15 DNA Purification Kit (MoBio, Carlsbad, CA) into 20 μ l, and diluted to 200 μ l with distilled sterilized water. Two μ l of the diluted PCR product was used as template in a nested PCR reaction, using the same conditions as above, except that primer *nifHr269* (5' CCG GCC TCC TCC AGG TA) was used as reverse primer instead of *nifHr*. Primer *nifHr269* was designed using a database that contained about 300 606-bp *nifH* gene fragments of uncultured root nodule populations and pure cultures (Mirza *et al.*, 2009; Welsh *et al.*, 2009), checked for

specificity in public databases and evaluated for amplification on 20 pure cultures representing different host infection groups. Sub-samples of the PCR reactions (5 µl) were checked for amplification products by gel electrophoresis (1% agarose in TAE buffer, wt/vol) after staining with ethidium bromide (0.5 µg ml⁻¹) (Sambrook *et al.*, 1989).

Sequence analysis: The amplicon of the nested PCR was cleaned with Ultra Clean 15 DNA Purification Kit, ligated into pGEM-T Easy plasmid (Promega, Madison, WI), as recommended by the manufacturer, and the construct transformed into *Escherichia coli* TOP-10 (Stratagene, Cedar Creek, TX). At least forty colonies from each transformation were selected and grown in LB medium supplemented with Ampicillin, and analyzed for the presence of a fragment of approx. 260 bp by PCR amplification with primers seqF (5' TCA CAC AGG AAA CAG CTA TGA C) and seqR (5' CGC CAG GCT TTT CCC AGT CAC GAC). Amplified fragments were purified using the Ultra Clean 15 DNA Purification Kit and sequenced using the CEQ 8800 Quickstart Kit according to the manufacturer's instructions (Beckman Coulter, Fullerton, CA) with the addition of 5% DMSO to the reaction mix. The sequencing reaction consisted of an initial incubation at 76°C for 5 minutes followed by 76°C for 5 minutes during which primers seqF or seqR and master mix were added, a subsequent incubation at 94°C for 2 minutes, and 35 cycles of temperature cycling (94°C for 30 seconds, 50°C for 30 seconds and 60°C for 4 minutes) and a final extension at 60°C for 10 minutes (Kukanskis *et al.*, 1999). Sequences were analyzed on a CEQ 8800 sequencer (Beckman Coulter) and deposited at Genbank under accession numbers GQ141268 - GQ141513.

Phylogenetic analyses: Sequences of *nifH* gene fragments obtained from soil clones (n=247), root nodules of *M. pennsylvanica* (n=121) and pure cultures of *Frankia* (n=48) were trimmed to be 252 bp long and aligned using Sequencher 4.2.2 (Gene Codes Corp., Ann Arbor, MI), CLUSTAL X and MacClade 4.05 (Maddison & Maddison, 1999; Thompson *et al.*, 1997) and analyzed using maximum parsimony (MP), neighbor joining (NJ), Bayesian and maximum likelihood (ML) methods (Figure. 5.1) as outlined in detail in (Welsh *et al.*, 2009). Briefly, MP and NJ methods were used in PAUP*4.0b10 with 10,000 bootstrap (BS) replicates (Swofford, 2002). Only those BS values of 70% or greater represent well supported nodes and thus only those were retained (Hillis & Bull, 1993). Bayesian and ML analyses were performed on the computer cluster of the ‘CyberInfrastructure for Phylogenetic RESearch’ project (CIPRES, www.phylo.org) using MrBayes (version 3.1.2) (Huelsenbeck & Ronquist, 2001) and RAxML-VI-HPC (Version 7.0.4) (Stamatakis, 2006b), respectively. Bayesian analyses included Metropolis-coupled Markov chain Monte Carlo (MCMCMC) sampling, a GTR+I+G model setting, 5 million generations and sampling every 1000 trees (Huelsenbeck & Ronquist, 2001). A 95% majority rule consensus tree for the Bayesian output of posterior probabilities (PP) was created in PAUP* with the first 500 trees removed as burn-in (Huelsenbeck *et al.*, 2002; Swofford, 2002). ML analyses included GTR+CAT approximation for rate heterogeneity (Stamatakis, 2006a), invariant sites, empirical base frequencies and 500 bootstrap replicates.

Sequences were assigned to specific clusters based on known genomic groupings, corresponding sequence similarity (95-99%) (Mirza *et al.*, 2009; Welsh *et al.*, 2009), general tree topology, and BS and PP values higher than 70% (Hillis & Bull, 1993) and

95% (Huelsenbeck *et al.*, 2002), respectively, as outlined in (Welsh *et al.*, 2009).

TreeView (Win16) was used to display the treefiles (Page, 1996) and MEGA version 4 was used to collapse branches (Tamura *et al.*, 2007) (Figure. 5.1). For presentation purposes this dataset of sequences from 48 pure cultures, 121 root nodule and 247 soil clones was reduced and reanalyzed independently by soil for direct comparisons of the diversity of frankiae in that soil and in root nodules formed using plant bioassays with *M. pennsylvanica* (Figure. 5.2, 5.3, 5.4).

Results and discussion

Amplicons of the appropriate size of about 260 bp were obtained by nested PCR from nucleic acid extracts from all six soils, and used to generate gene clone libraries. All clones (n=40-43 per library) that were selected at random from each library harbored partial *nifH* gene sequences of frankiae as demonstrated by BLAST and FASTA searches in Genbank and EMBL, respectively (Altschul *et al.*, 1997; Pearson & Lipman, 1988) which demonstrated a high specificity of the primer combination nifHf1/nifHr269 for the *nifH* gene of the genus *Frankia*. Sequences with similarity to frankiae of the *Elaeagnus* and the *Alnus* host infection groups, including those similar to *Casuarina*-infective frankiae, were amplified which suggested non preferential amplification or absence of primer biases toward a particular group of *Frankia* strains.

Meta-analyses of a total of 416 sequences that included those of the 247 soil clones, as well as sequences of 48 pure cultures and of uncultured frankiae of 121 root nodules of *M. pennsylvanica* that were trimmed to 252 bp to get a uniform length of all sequences revealed an overall diversity with four and six clusters of frankiae representing the *Elaeagnus* (EI - EIV) and the *Alnus* (AI - AVI) host infection groups, respectively, when

96 to 97% similarity thresholds were used (Figure. 5.1). This clustering was consistent regardless of the phylogenetic method used (data not shown), and resembled that described in our previous studies (Mirza *et al.*, 2009; Welsh *et al.*, 2009). Four of these clusters (i.e., EI, EIII, AIII, and AVI) were not represented by any pure cultures of *Frankia* which impacts the accurate assignment of these clusters to a host infection group. In previous analyses, uncultured frankiae from root nodules of *M. pennsylvanica* in clusters EI and EIII were well supported as being included in the *Elaeagnus* host infection group (Mirza *et al.*, 2009; Welsh *et al.*, 2009). Frankiae of clusters AIII and AVI were not included in that partition, and thus were assumed to be in the *Alnus* host infection group (Welsh *et al.*, 2009). Lack of strong support for the assignment of these clusters in this analysis is likely due to the reduction in sequence information when 252 bp sequences were used instead of 522 bp sequences. However, the overall topology and specific grouping of these clusters is consistent with previous analyses (Mirza *et al.*, 2009; Welsh *et al.*, 2009), and thus does not impair the assignment of these clusters to the host infection groups.

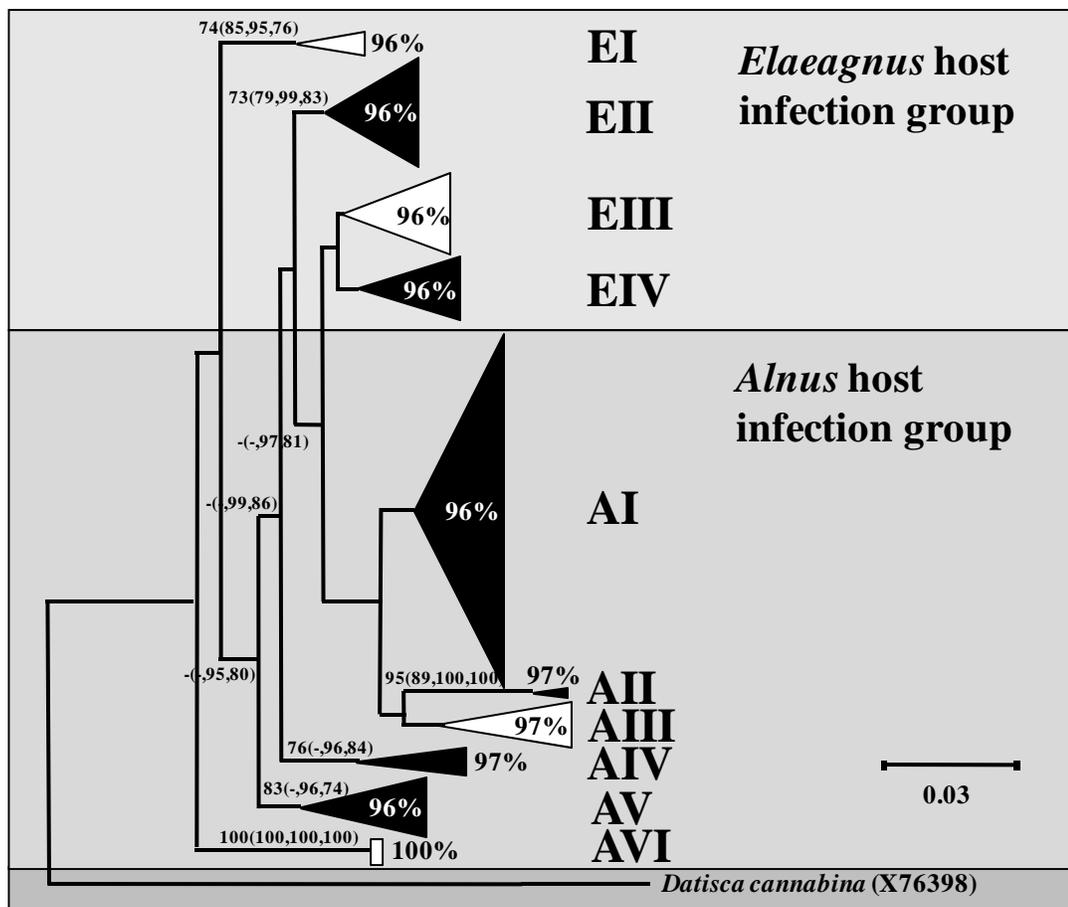


Figure 5.1: Diversity of uncultured *Frankia* populations in root nodules of *Morella pensylvanica* and soils clones from six sites in five continents. Maximum likelihood-based tree generated using 252 bp of the *nifH* gene of 247 clones from six libraries generated from soils from six sites in five continents, of 121 uncultured *Frankia* populations from root nodules of *Morella pensylvanica* inoculated with these soils (Mirza *et al.*, 2009; Welsh *et al.*, 2009), and of 48 pure cultures of *Frankia*. Branches were collapsed at key nodes based on topology and bootstrap (BS) support values to give an overall picture of the diversity of *Frankia* recovered. Numbers at nodes reflect maximum likelihood BS support measures and numbers in parentheses represent BS measures and posterior probabilities (PP) from neighbor joining, Bayesian, and maximum parsimony analyses, respectively. The outgroup was a sequence from an uncultured *Frankia* population from nodules of *Datisca cannabina* (X76398). Cluster designations on the right represent *Frankia* of the *Elaeagnus* host infection group (EI - EIV) or the *Alnus* host infection group (AI - AVI). Black triangles represent clusters where sequences of pure cultures were available, and white triangles those with no sequence information for pure cultures available. Numbers within or beside the triangles represent the percent similarity of sequences in that cluster.

Overall, about 36% of the sequences were assigned to frankiae of the *Elaeagnus* host infection and 64% to frankiae of the *Alnus* host infection group (Table 1). Although much shorter *nifH* gene sequences (i.e., 252 bp) were used for the analyses compared to previous studies (552 bp and longer) (Mirza *et al.*, 2009; Welsh *et al.*, 2009), the assignment of sequences to the *Elaeagnus* or *Alnus* host infection groups was generally identical between studies. However, small shifts were observed in assignments of sequences within the *Elaeagnus* host infection group (including uncultured root nodule populations as well as strain Cc1.17) (data not shown). Overall, the assignment of about 95% of the sequences representing uncultured populations in root nodules and pure cultures reduced in size to 252 bp was congruent with their previously published grouping based on 552 bp *nifH* gene fragments (Mirza *et al.*, 2009; Welsh *et al.*, 2009). This result suggests that the reduction in size of *nifH* gene sequences did not impact basic phylogenetic analyses and sequences could reliably be used to compare different *Frankia* populations in our meta-analysis (Figure. 5.1).

Table 5.1: Percentage of *nifH* gene sequences identifying *Frankia* of the *Elaeagnus* or *Alnus* host infection groups, from the root nodules of *Morella pensylvanica* and soils clones from five continents. Percentage of *nifH* gene sequences identifying *Frankia* of the *Elaeagnus* or *Alnus* host infection groups, respectively, in nodules formed on *Morella pensylvanica* after inoculation with slurries of soil obtained from different countries (= nodules), or in clone libraries generated from DNA extracted from the same soils (= soil).

Soil origin	<i>Elaeagnus</i> host infection group		<i>Alnus</i> host infection group	
	Nodules	soil	nodules	Soil
Hungary	35 (n=7)	7 (n=3)	65 (n=13)	93 (n=40)
Peru	95 (n=19)	39 (n=16)	5 (n=1)	61 (n=25)
Rwanda	70 (n=14)	7 (n=3)	30 (n=6)	93 (n=40)
Japan	60 (n=12)	40 (n=16)	40 (n=8)	60 (n=24)
USA (Alaska)	5 (n=1)	78 (n=31)	95 (n=19)	22 (n=9)
USA (Wisconsin)	57 (n=12)	0	43 (n=9)	100 (n=40)

The numbers in parentheses represent the number of sequences from nodules or soil clone libraries assigned to the respective host infection group

Compared to the meta-analysis, however, the reduction in the number of sequences used to compare frankiae in soil and nodules from one site (Figures. 5.2, 5.3, 5.4) resulted in inconsistencies of the phylogenetic position of individual pure cultures (ARgP5, AgKG'84/4 and Cc1.17). These inconsistencies were attributed to long branch attraction, i.e., that in the absence of closely related in-group sequences, the long branches on a tree tend to deviate from their original position and cluster together (Hendy & Penny, 1989).

Possibilities for corrections of the phenomenon include the use of multiple methods of phylogenetic analyses, the addition of closely related in-group sequences to long branch taxa, and the exclusion of long branch taxa from analyses (Dacks *et al.*, 2002; Farias *et al.*, 2001; Hanelt *et al.*, 1996). Since strains ARgP5, AgKG'84/4 and Cc1.17 are relatively unique with no or only few other isolates closely related, the most commonly used method to avoid long-branch attraction, i.e., the addition of sequences closely related to long-branch taxa (Hendy & Penny, 1989; Swofford *et al.*, 1996) could not be performed. Since all phylogenetic analyses methods, including maximum likelihood analyses that is least sensitive but not completely immune to long branch attraction (Farris, 1999; Lockhart *et al.*, 1996), can suffer from long branch attraction, we usually accepted the inconsistency of their position (strains ARgP5 and AgKG'4/4 in Figures. 5.2, 5.3, 5.4). In one case, strain Cc1.17 was removed from the analyses (i.e., from analyses of soil from Peru; Figure. 5.2) in order to maintain accurate host infection group partitions even though the simple exclusion of the long-branch taxa from the analyses reduced the phylogenetic information content (Bergsten, 2005).

Comparative sequence analyses of all selected clones from the six libraries (n=247 clones) using 96 to 97% similarity thresholds revealed the presence of three and four clusters of frankiae representing the *Elaeagnus* and the *Alnus* host infection groups, respectively (Figures. 5.2, 5.3, 5.4). Diversity of frankiae was represented by fewer clusters (i.e., up to four in total) within individual libraries, with one cluster generally harboring the vast majority of sequences. Rarefaction analyses using these cluster assignments therefore demonstrated that a sufficient number of sequences had been analyzed for complete coverage of diversity of frankiae in soil (data not shown). The

most prominent cluster, cluster AI from the *Alnus* host infection group, that represented 61% of the sequences retrieved from the soil clone libraries was detected in all libraries although in highly variable frequencies with extremes covering 100% of the sequences obtained (i.e., soil from Wisconsin) or only 5% of the sequences (i.e., soil from Japan) (Table 2). Most of the pure cultures isolated from different regions of the world including sites in North America, Asia and Europe also clustered in this group which indicates a wide distribution of these frankiae in nature which suggests that they do not require continuous symbiotic interaction with specific host plants (Benson & Dawson, 2007).

Table 5.2: Percentage of *nifH* gene sequences identifying *Frankia* of subgroups within the *Alnus* host infection group, from the root nodules of *Morella pensylvanica* and soils clones from five continents. Percentage of *nifH* gene sequences identifying *Frankia* of subgroups within the *Alnus* host infection group in nodules formed on *Morella pensylvanica* after inoculation with slurries of soil obtained from different countries (= nodules), or in clone libraries generated from DNA extracted from the same soils (= soil).

Soil origin	AI (ArI3)*		AII (AgKG'84/4)*		AIII (none)*		AIV (CjI-82)*		AV (Ag45/Mut15)*		AVI (none)*	
	nodules	soil	Nodules	soil	nodules	soil	nodules	soil	nodule	Soil	nodules	soil
Hungary	50 (n=10)	91 (n=39)	0	0	0	0	15 (n=3)	0	0	2 (n=1)	0	0
Peru	5 (n=1)	61 (n=25)	0	0	0	0	0	0	0	0	0	0
Rwanda	0	89 (n=38)	0	0	5 (n=1)	2 (n=1)	0	0	0	2 (n=1)	25 (n=5)	0
Japan	0	5 (n=2)	0	0	30 (n=6)	40 (n=16)	5 (n=1)	15 (n=6)	5 (n=1)	0	0	0
Alaska	5 (n=1)	15 (n=6)	0	0	0	0	0	0	90 (n=18)	8 (n=3)	0	0
Wisconsin	43 (n=9)	100 (n=40)	0	0	0	0	0	0	0	0	0	0

The numbers in parentheses represent the number of sequences from nodules or soil clone libraries assigned to the respective cluster within the *Alnus* host infection group

*representative of pure cultures of *Frankia* associated with this subgroup

Comparative analyses of sequences retrieved from soil clone libraries and root nodules formed by frankiae on *M. pennsylvanica* after inoculation with the same soil revealed that sequences most abundant in clone libraries were not quantitatively representing *Frankia* populations forming nodules. On the host infection group level, for example, about 93% and 61% of the sequences obtained from clone libraries generated from nucleic acid extracts from soils from Hungary and Peru, respectively, were assigned to the *Alnus* host infection group, while only 65% and 5% of the sequences obtained from root nodules formed after inoculation with these soils were in this group (Figure. 5.2; Table 1). Similar differences were obtained for the other soils from Rwanda, Japan, Alaska, and Wisconsin (Figures. 5.3, 5.4; Table 1). These discrepancies were even more pronounced for specific clusters within these groups (Tables 2, and 3). For example, 65% of all sequences in nodules obtained after inoculation with soil from Rwanda represented frankiae of the *Elaeagnus* host infection group cluster E1, but none of the sequences from the soil clone library was from this group (Figure. 5.3; Table 3). Similarly, 90% of the sequences from root nodules obtained after inoculation with the soil from Alaska represented frankiae of the *Alnus* host infection group cluster AV, but only about 7% of soil clones were from this group (Figure. 5.4; Table 2). These results demonstrate large differences in cluster assignments between sequences retrieved from soil clone libraries and those obtained from nodules, with assignments to the same cluster only rarely encountered for individual soils.

Table 5.3: Percentage of *nifH* gene sequences identifying *Frankia* of subgroups within the *Elaeagnus* host infection group, from the root nodules of *Morella pensylvanica* and soils clones from five continents. Percentage of *nifH* gene sequences identifying *Frankia* of subgroups within the *Elaeagnus* host infection group in nodules formed on *Morella pensylvanica* after inoculation with slurries of soil obtained from different countries (= nodules), or in clone libraries generated from DNA extracted from the same soils (= soil).

Soil origin	EI (none)*		EII (EAN1pec)*		EIII (none)*		EIV (Cc1.17)*	
	nodules	soil	nodules	Soil	nodules	soil	nodules	Soil
Hungary	0	0	0	0	0	2 (n=1)	35 (n=7)	5 (n=2)
Peru	0	0	35 (n=7)	7 (n=3)	60 (n=12)	32 (n=13)	0	0
Rwanda	65 (n=13)	0	5 (n=1)	7 (n=3)	0	0	0	0
Japan	25 (n=5)	0	35 (n=7)	0	0	10 (n=4)	0	30 (n=12)
Alaska	0	0	0	75 (n=30)	5 (n=1)	0	0	2 (n=1)
Wisconsin	0	0	0	0	33 (n=7)	0	24 (n=5)	0

The numbers in parentheses represent the number of sequences from nodules or soil clone libraries assigned to the respective cluster within the *Elaeagnus* host infection group

*representative of pure cultures of *Frankia* associated with this subgroup

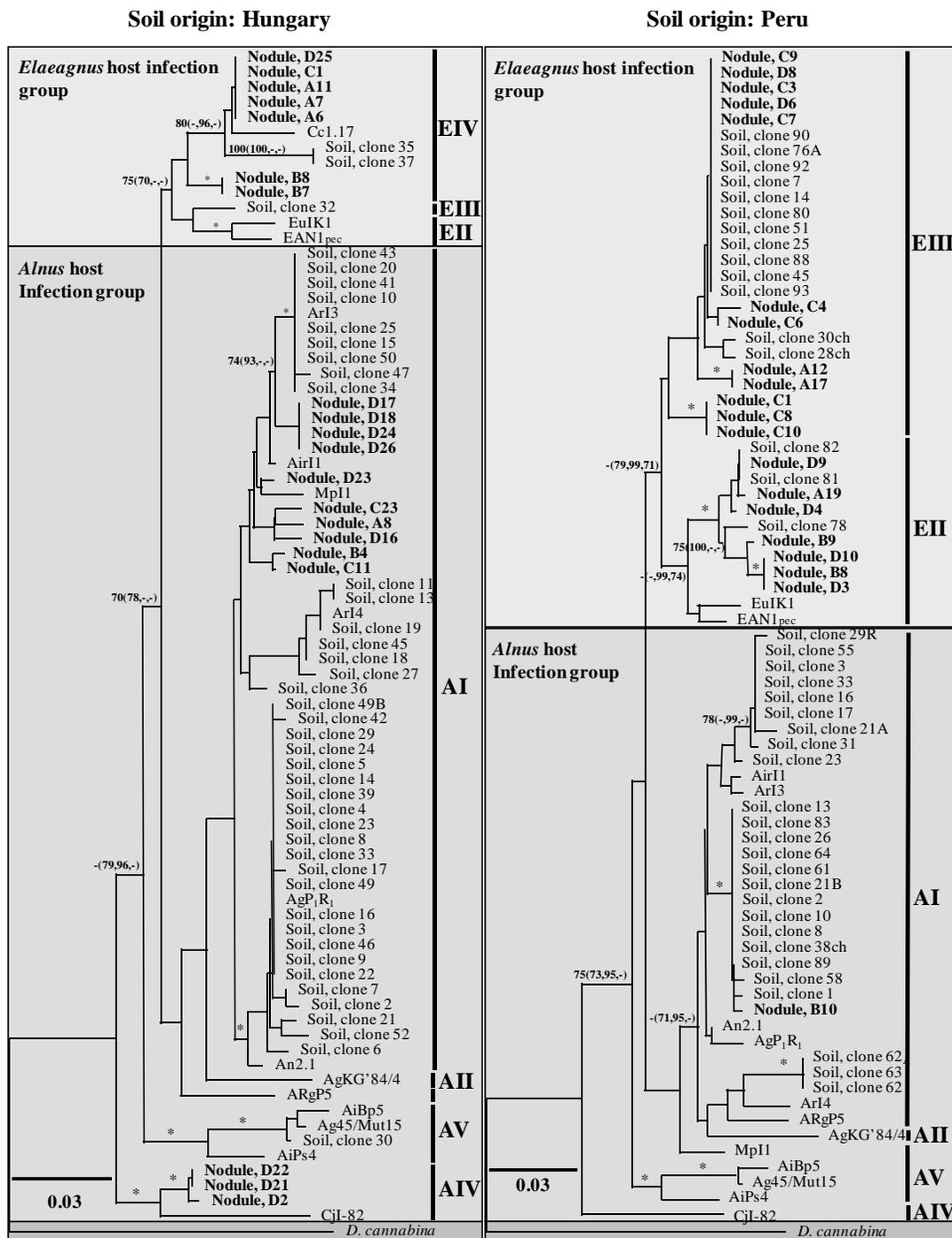


Figure 5.2: Diversity of uncultured *Frankia* populations in root nodules of *Morella pensylvanica* and soils clones from Hungary and Peru. Maximum likelihood-based trees generated using 252 bp *nifH* gene fragments obtained from soil clone libraries (Soil, clone) generated from soils from Hungary (left panel) and Peru (right panel), of pure cultures of *Frankia* and of uncultured *Frankia* populations from root nodules of

Figure 5.2 continued. *Morella pensylvanica* inoculated with these soils (Nodule, in bold) (Welsh *et al.*, 2009). Cluster designations on the right represent frankiae of the *Elaeagnus* host infection group with cluster acronyms EI to EIV, sequences assigned to the *Alnus* host infection group with cluster acronyms AI to AVI. Numbers reflect maximum likelihood bootstrap support measures (BS) and numbers in parentheses represent BS measures and posterior probabilities (PP) from, neighbor joining, Bayesian, and maximum parsimony analyses, respectively. Asterisks at tip nodes reflect high BS or PP values from at least three of the four phylogenetic methods utilized in these analyses. The outgroup was a sequence from an uncultured *Frankia* population from nodules of *Datisca cannabina* (X76398).

Figure 5.3 continued. cultures of *Frankia* and of uncultured *Frankia* populations from root nodules of *Morella pensylvanica* inoculated with these soils (Nodule, in bold) (Welsh *et al.*, 2009). Cluster designations on the right represent frankiae of the *Elaeagnus* host infection group with cluster acronyms EI to EIV, sequences assigned to the *Alnus* host infection group with cluster acronyms AI to AVI. Numbers reflect maximum likelihood bootstrap support measures (BS) and numbers in parentheses represent BS measures and posterior probabilities (PP) from neighbor joining, Bayesian, and maximum parsimony analyses, respectively. Asterisks at tip nodes reflect high BS or PP values from at least three of the four phylogenetic methods utilized in these analyses. The outgroup was a sequence from an uncultured *Frankia* population from nodules of *Datisca cannabina* (X76398).

Figure 5.4 continued. (Soil, clone) generated from soils from USA, Alaska (left panel) and Wisconsin (right panel), of pure cultures of *Frankia* and of uncultured *Frankia* populations from root nodules of *Morella pensylvanica* inoculated with these soils (Nodule, in bold) (Mirza *et al.*, 2009; Welsh *et al.*, 2009). Cluster designations on the right represent frankiae of the *Elaeagnus* host infection group with cluster acronyms EI to EIV, sequences assigned to the *Alnus* host infection group with cluster acronyms AI to AVI. Numbers reflect maximum likelihood bootstrap support measures (BS) and numbers in parentheses represent BS measures and posterior probabilities (PP) from neighbor joining, Bayesian, and maximum parsimony analyses, respectively. Asterisks at tip nodes reflect high BS or PP values from at least three of the four phylogenetic methods utilized in these analyses. The outgroup was a sequence from an uncultured *Frankia* population from nodules of *Datisca cannabina* (X76398).

Several reasons might be considered for the detection of largely different *Frankia* populations in soil and in root nodules formed after inoculation with the same soil based on comparative sequence analysis of *nifH* gene fragments retrieved from root nodules or from clone libraries. The host plant species, for example, has been shown to have a large effect on the selection of *Frankia* strains from soil for potential nodule formation (Mirza *et al.*, 2009). Diversity of frankiae of the *Elaeagnus* host infection group was larger in nodules on *M. pensylvanica* and also *M. cerifera* that are both supposed to be promiscuous for frankiae, i.e., it forms nodules with frankiae belonging to both the *Elaeagnus* and the *Alnus* host infection groups (Baker, 1987; Dobritsa *et al.*, 1990; Huang *et al.*, 1985) compared to different *Elaeagnus* species (Mirza *et al.*, 2009). None of the plant species, however, captured the entire diversity of nodule-forming frankiae and the distribution of clusters of *Frankia* populations and their abundance in nodules was unique for each of the plant species with only one cluster being ubiquitous and most abundant while the remaining clusters were only present in nodules of one or two host plant species (Mirza *et al.*, 2009). These results demonstrate a significant effect of the plant species on the selection of frankiae from soil for nodulation.

However, it has been shown that gene clone libraries do not necessarily provide an accurate picture of diversity and abundance of microorganisms in the environment (Chandler *et al.*, 1997; Cottrell & Kirchman, 2000; Timke *et al.*, 2005), even though the contrary has been shown as well (Cary *et al.*, 1997; Schramm *et al.*, 1998; Tonolla *et al.*, 2004). The composition of sequences in libraries might be affected by PCR errors producing sequence artifacts, and/or by PCR and cloning bias resulting in a skewed distribution of sequences in these libraries (Acinas *et al.*, 2005). Clustering the sequences into sequence similarity groups is a way to constrain sequence artifacts. Analyses of 16S rRNA genes demonstrated that a 99% similarity value accounted for these artifacts (Acinas *et al.*, 2005), and thus our clustering using 96 to 97% similarity values should be sufficient to circumvent potential problems of small sequence artifacts. The clustering also confirmed the assignment of all sequences from soil clone libraries to clusters established by comparative analyses of sequences retrieved from pure cultures or uncultured nodule populations (Mirza *et al.*, 2009; Welsh *et al.*, 2009) and thus confirming low PCR bias with respect to chimera production or non-specific primer binding. The retrieval of low diversity of frankiae from all soil clone libraries might therefore be due to preferential amplification of more abundant sequences in these soils (Chandler *et al.*, 1997), and thus reflect an accurate assessment of the most abundant *Frankia* populations in these soils.

Assuming that our *nifH* gene clone library analyses retrieved sequences of the most abundant *Frankia* populations in these soils, the result that populations in root nodules of *M. pennsylvanica* generally do not match these populations suggests that nodule formation reflects the effect of the plant species on a specific *Frankia* population rather than

representing a quantitative picture of the overall structure of nodule-forming populations in soil. The correlation between cell numbers in soil and nodule formation, however, remains problematic because a nodule can theoretically be induced by a single spore, a hyphal fragment or a colony (Mirza *et al.*, 2009). This problem might be magnified in bioassays used to quantify nodule-forming frankiae because these neglect non-nodulating as well as non-competitive *Frankia* populations, as well as any effects of sample processing (e.g., the disruption of large filaments into smaller units) on the *Frankia* populations in soil. In order to avoid potential effects of experimental conditions, comparative analyses of soil and root nodule populations of frankiae should therefore include analyses of populations in natural stands of actinorhizal plants. Although initial studies have shown that *Frankia* strains can grow saprotrophically in filamentous form in the rhizosphere of host and non-host plants, and some even with leaf litter as a nutrient resource (Mirza *et al.*, 2007), it is currently unknown whether the infectious *Frankia* particles in soil are really actively growing organisms present in a vegetative form (i.e., in long filaments as in pure culture, in short fragments or in single cell form) or as spores activated by exudates of the host plant. Future studies therefore need to expand investigations on saprophytic growth of frankiae in soil and analyze potential effects of the growth state and occurrence of frankiae in soil on root nodule forming capacity.

Acknowledgements

The authors are indebted to the Texas State Department of Biology and the National Science Foundation (GK-12 grant No. 0742306). Support for MWP and JPR was provided by the Warner College of Natural Resources at CSU. We are grateful for the assistance of C. Herron, T. Hoelzle, J. Holden, J. Johnson, M. Kizlinski, L.G. Perry, M. Schultz, K. Torok, and H. Yasudain in collecting soil samples.

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CHAPTER VI

GENERAL DISCUSSION

The work presented in this Ph.D. dissertation focused on two basic objectives: 1) to elucidate the effects of specific environmental conditions on the fate of introduced *Frankia* strains in soil microcosms, and 2) to highlight the limitations of plant bioassay analyses in describing the diversity of *Frankia* in soil. Work on the first objective provided evidence that *Frankia* strains differ with respect to their ability to utilize specific organic compounds (chapter II, and III), and that -within the *Alnus* host infection group- the utilization of leaf litter is a specific trait of few *Frankia* strains, and reflected in their taxonomic position (chapter III).

Specific results for the first objective were that *Frankia* strains have the potential to grow saprotrophically, with the majority of strains belonging the *Elaeagnus* and *Alnus* host infection groups growing in the rhizosphere of a non-host plant, *Betula pendula*, but not in the surrounding bulk soil (Mirza, *et al.*, 2009a). *Casuarina*-infective strains that are generally assigned to the *Alnus* host infection group (Mirza, *et al.*, 2009b; Welsh, *et al.*, 2009), however, did not grow in the rhizosphere of *B. pendula*, even though these same strains did grow in the rhizosphere of *Casuarina cunninghamiana*. In contrast to results obtained for the rhizosphere of *B. pendula*, saprotrophic growth on leaf litter as a C source was restricted to a small fraction of *Frankia* strains that all

belonged to a distinct phylogenetic cluster within the *Alnus* host infection group. These results demonstrated that saprotrophic growth of frankiae was a common trait for most members of the genus, and the supporting factors for growth (i.e. carbon utilization capabilities) varied with host infection group and phylogenetic affiliation of the strains. These studies also provided information on the usefulness of comparative *nifH* gene sequences analyses to distinguish *Frankia* clusters within the *Elaeagnus* and *Alnus* host infection group, with comparable assignments of strains but better resolution than the previously used insertion in the 23S rRNA gene.

While these studies have provided significant baseline information on the ability of *Frankia* strains to grow in the rhizosphere or with leaf litter, the potential consequences of these physiological differences on root nodule formation on a host plant remain unresolved. Growth in soil -in addition to growth in the rhizosphere- seems to indicate an ecological advantage, i.e. an increase in biomass compared to strains that are unable to grow. However, since *Frankia* shows explicit tip growth as demonstrated in chapters II, and III, which results in the elongation of filaments without changes in numbers of filaments, the increase in biomass does not result in an increase in nodulation units. This might be different for those strains that do grow in the rhizosphere, but not with leaf litter. Without plants, these strains might become dormant, with filaments disintegrating into smaller fragments or cells even forming spores. This scenario seems plausible because filaments of non-growing strain remain detectable after staining with DAPI which intercalates into DNA. However, they were undetectable by *in situ* hybridization likely due to the reduced amount of rRNA which is a typical situation for dormant stages of cells. Resuscitation of these dormant cells in

the rhizosphere of a host or non-host plant -though at low biomass- would result in a large increase of nodulation units, and thus provide these non-growing strains with an advantage for nodule formation. This scenario would contradict previous assumptions that “the nodulating ability of *Frankia* is controlled largely by the physiological status of *Frankia*, as indicated by infectivity, rather than total population size” (Myrold & Huss-Danell, 1994). Thus, future studies need to incorporate investigations on nodule forming capacity of these strains as well as the accurate identification of dormant stages to elucidate consequences of growth or non-growth of *Frankia* populations in soil with leaf litter.

The experimental setup could follow that outlined in chapters II and III, with samples subjected to bioassays directly after inoculation, and after incubation for 6 weeks. The potential outcome could be an identical nodulation capacity of the growing strains at both times, while the non-growing strains would exhibit higher nodulation capacity after incubation for 6 weeks. Strains could easily be identified in nodules formed by fingerprinting techniques such as rep-PCR. This scenario would assume an activation of the dormant stages of the non-growing strains in the rhizosphere of the host plant. Additional proof for activation could be retrieved by resuscitation studies using microcosms after 6 weeks of incubation, and either planting them to provide root exudates (Krumholz, *et al.*, 2003), or directly adding C sources such as propionate (Caru, *et al.*, 1997), both of which should result in activation and growth of dormant cells which could easily be visualized by *in situ* hybridization. Detection of growth could provide some insight on dormant stages of frankiae in soil, and help explain the detection of infective strains from environments not considered to be adequate habitats

for frankiae, such as river and lake sediments (Huss-Danell, *et al.*, 1997), partially decomposed wood (Li, *et al.*, 1997), deep soil horizons (Nalin, *et al.*, 1997), or soils stored long-term (up to 12 years) (Chaia, *et al.*, 2007).

All studies on saprotrophic growth of frankiae in soil presented in this dissertation focused on the most prominent environmental factor meant to affect growth of frankiae in soil, i.e. C availability in the form of organic material in general, and as plant root exudates (Rönkkö, *et al.*, 1993) or as leaf litter (Nickel, *et al.*, 2001) in particular. It is known, however, that many other factors might affect growth or growth rate of *Frankia* in soil because the nodulation capacity of soil is affected by the soil pH (Griffiths & McCormick, 1984; Jaman, *et al.*, 1992; Roller, *et al.*, 1992; Crannell, *et al.*, 1994), the soil matric potential (Schwintzer, 1985; Dawson, *et al.*, 1989; Nickel, *et al.*, 1999), the soil organic matter content (Nickel, *et al.*, 2001; Zimpfer, *et al.*, 2003), and the availability of elements such as nitrogen (Kohls & Baker, 1989; Thomas & Berry, 1989) or phosphorus (Sanginga, *et al.*, 1989; Yang, 1995). Additional studies on the effects of these environmental factors individually as well as in combination are therefore necessary to elucidate whether these soil conditions affect the nodulation process or the survival of *Frankia* in soil (Benson & Silvester, 1993). The combination of a large variety of environmental factors –and the inclusion of seasonal variability– could ultimately result in conditions that largely resemble those of their natural habitat and thus provide information on the fate of these organisms under natural conditions, i.e. whether they can grow continuously as increase in filament under the favorable growth conditions and makes spore under the extreme soil conditions (Mirza, *et al.*, 2007), or may grow in the form of annual cycles i.e., spore germination, growth and

sporulation corresponding to the active plant growth stages, or may get amplified in the root nodules and subsequently released into the environment after the senescence of the root nodules (Van Dijk, 1979; Benson & Silvester, 1993).

While these additional studies are definitely needed to analyze the potential activation of *Frankia* cells in the rhizosphere of host or non-host plants and to assess the consequences of activation for nodule formation in competition with growing frankiae, the ecological consequences also need to be assessed in less defined systems (e.g., greenhouse experiments covering the entire season), and ultimately be evaluated in a natural setting with either introduced strains or indigenous populations. Such studies, however, would require the availability of extremely sensitive analysis methods that would allow for qualitative and quantitative studies on overall and specific *Frankia* populations in soil. The usefulness of *in situ* hybridization on indigenous populations of *Frankia* in soils is largely reduced because soils represent highly heterogeneous environments with a tremendous diversity of organisms (Torsvik, *et al.*, 1990; Torsvik, *et al.*, 1996; Dunbar, *et al.*, 2002; Gans, *et al.*, 2005) and with frankiae present in low numbers (approximately 10^4 - 10^5 cells g^{-1} soil) (Hahn, *et al.*, 1990; Myrold, *et al.*, 1994b) as part of a large microbial community (more than 10^9 cells g^{-1} soil) (Zarda, *et al.*, 1997; Chatzinotas, *et al.*, 1998).

Although PCR-based methods, distinctive target genes (e.g., 16S rRNA gene), and specific primers are available for the detection of frankiae on the genus level (Hahn, *et al.*, 1999), the use of these detection systems is still impacted by the low abundance of frankiae in soil and the limited resolution of phylogenetically relevant target genes such as rRNAs for assessing diversity within the genus. Current studies on the

diversity of frankiae in root nodules of different host plant species and those formed by frankiae from different soils have provided an alternative to rRNA genes as target or template in detection assays. In recent studies we used comparative sequence analyses of *nifH* gene fragments to distinguish *Frankia* populations in root nodules formed by frankiae from different soils (Mirza, *et al.*, 2009b, Welsh, *et al.*, 2009), and to analyze the diversity of *Frankia* populations from the same soil forming nodules on different host plant species (Mirza, *et al.*, 2009a). These studies provided information on nodule-forming frankiae in different soils and on the effect of plant species in bioassays on these populations, but also build up a large database of sequences from uncultured frankiae with more than 400 sequences that -in addition to sequences from pure cultures that were used to classify the uncultured frankiae taxonomically- allowed us to differentiate several clusters of frankiae within the *Elaeagnus* and *Alnus* host infection groups (Mirza, *et al.*, 2009a; Mirza, *et al.*, 2009b ; Welsh, *et al.*, 2009). This database provide baseline data for the design of probes and primers targeting frankiae on different levels, i.e. the genus level, the host infection group level or even more specific levels of detection (i.e. cluster level) that would allow to analyze specific populations such as, for example, those growing in the presence of leaf litter.

Quantitative methods such as *qPCR* are available for many other organisms (Stults, *et al.*, 2001; Tajima, *et al.*, 2001; Okano, *et al.*, 2004; Skovhus, *et al.*, 2004), however, need to be implemented and verified for frankiae.

Results for the second objective demonstrated that the host plant species has a large effect in the selection of *Frankia* strains from soil for potential nodule formation (chapter IV), and that this effect results in large differences between *Frankia*

populations detected directly in soil and those in root nodules (chapter V).

Consequently, the choice of the capture plant species has a significant effect in bioassays on diversity estimates of frankiae in soil. Diversity estimates of frankiae that include gene clone library analyses are equally impacted because only the most abundant *Frankia* populations will be detected. It is therefore not surprising that comparative analyses between sequences retrieved from clone libraries and those obtained from nodules displayed large differences in cluster assignments, with assignments to the same cluster only rarely encountered for individual soils. These results demonstrated large differences between detectable *Frankia* populations in soil and those in root nodules indicating the inadequacy of bioassays for the analysis of frankiae in soil and the role of plants in the selection of frankiae from soil for root nodule formation.

Since most studies on *Frankia* populations in soil -with a few exceptions- were based on plant bioassays, i.e., specific capture plants were inoculated with soil and subsequently analyzed for root nodules using different targets such as the 16S rRNA gene (Mirza, *et al.*, 1994; Clawson, *et al.*, 1997; Ritchie & Myrold, 1999), the *gln-II* gene (Clawson, *et al.*, 2004; Gtari, *et al.*, 2004) or the *nifH* gene (Jeong, *et al.*, 1999; Gtari, *et al.*, 2007), or methods like Rep-PCR (Murry, *et al.*, 1995; Murry, *et al.*, 1997; Jeong & Myrold, 1999), PCR-RFLPs (Jamann, *et al.*, 1992; Nalin, *et al.*, 1997), or *in situ* hybridization (Maunuksela *et al.*, 1999), profound artifacts might have been retrieved rather than accurate pictures of *Frankia* diversity and abundance. Potential problems with artifacts might be magnified in any plant bioassay-based analyses because non-competitive *Frankia* populations, including those present in low numbers,

or competition for infection on the capture plant between different *Frankia* populations in soil cannot be assessed (Huss-Danell & Myrold, 1994; Maunuksela, *et al.*, 2000).

Thus, in order to describe the diversity of *Frankia* populations in soil, it is important to bypass the process of root nodule formation and to analyze *Frankia* populations directly in the soil.

NifH gene clone libraries generated from soil provided a means for this type of analysis, however, analyses were restricted by the limited sensitivity that allowed only to detect the most abundant populations. It was therefore not surprising that cluster assignments between sequences retrieved from soil clone libraries and those obtained from nodules formed by frankiae of the same soil did not match, suggesting that the partner choice is controlled by the plant species, and by the composition and competition of *Frankia* strains in soil and not by their abundance. In order to determine overall diversity of frankiae in soil accurately, alternatives to gene clone library analyses need to be assessed. One possibility could be based on pyrosequencing in which genus-specific *nifH* gene fragments of *Frankia* are retrieved from DNA extracted from soils and individually sequenced without cloning. Comparative analyses of diversity of frankiae determined in root nodules of capture plants, in gene clone libraries and in runs for pyrosequencing even at low coverage (i.e., about 5,000 sequences) should highlight the power of this new technology to analyze the entire community of frankiae in a particular soil provided specific primers targeting *nifH* gene fragments of frankiae are available.

Pyrosequencing DNA extracted from soil with specific primers will not only retrieve information on the diversity of frankiae, but also increase the database of

Frankia-specific *nifH* gene sequences significantly. Using this database to design *Frankia*-specific probes/primers for detection of populations on different level (i.e., genus, host infection group, clusters) and their subsequent use in quantitative analyses methods such as *qPCR* that is meant to be 100 times more sensitive than regular PCR and 1000 times more sensitive than *in situ* hybridization (Wawrik, *et al.*, 2002) will not only provide information on the abundance of a particular group of *Frankia* in soil but also tell us about their ecological function in that environment (Wawrik, *et al.*, 2002). Ecophysiological studies on indigenous *Frankia* populations might use this technique to assess effects of root exudates on growth and nodule-forming capacity of specific *Frankia* strains, and demonstrate potential effects of environmental characteristics on the fate of these frankiae, both under laboratory and field conditions.

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