Population dynamics of Frankia in soil

Diss. ETH No. 13731

Population dynamics of *Frankia* in soil

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

For the degree of

DOCTOR OF NATURAL SCIENCES

Presented by

ANJA MARTINA NICKEL

Dipl. Biol. Univ.

born May 2, 1970

from Germany

accepted on the recommendation of

Prof. Dr. J. Zeyer, examiner

Prof. Dr. A. Hartmann, co-examiner

Dr. R. Siegwolf, co-examiner

Contents

Summary

Seite Leer /
Blank leaf

Summary

Actinomycetes of the genus *Frankia* can form a symbiosis with actinorhizal plants, which leads to the formation of root nodules. Within the nodules numerous *Frankia* cells differentiate into vesicles in which molecular nitrogen is fixed. Actinorhizal plants, of which *Alnus* species (alders) play an important role in the Northern Hemisphere, are not only used for the production of timber, but also for reforestation and the improvement of soils. The N_2-fixing rate of the symbiosis is influenced by factors such as pH, drought or temperature, which must be considered when trying to improve the efficiency of the symbiosis.

Since *Frankia* strains are difficult to obtain in pure culture, molecular methods that are independent of cultivation have become increasingly important tools in *Frankia* research in recent years. Comparative sequence analysis of phylogenetically relevant macromolecules such as ribosomal RNAs (rRNA), for example, has been used to separate strains of *Frankia* forming a symbiosis with alders into 5 groups (I, IIIa, IIIb, IVa and IVb). The growing database of discriminative target sequences has resulted in the development of family- and group-specific primers and probes. By using them in PCR or in *in situ* hybridization, it is now possible to identify *Frankia* populations and consequently to study environmental influences on the dynamics of indigenous or introduced *Frankia* populations in plants and soil.

In this project, group-specific probes were used to study the competitive abilities of indigenous and introduced *Frankia* strains on the nodulation of *Alnus glutinosa* plants under different environmental conditions: (i) dry, nitrate-rich soil, and (ii) wet, nitrate-poor soil. Non-inoculated plants harbored only *Frankia* with distorted vesicles (group IIIa) or no *Frankia* cells at all. Plant leaves were slightly chlorotic, indicating that even the high level of nitrate in the dry soil was not providing optimal plant growth conditions. Nodules from inoculated plants always contained vesicle-producing *Frankia* (groups IIIa and IV). In both treatments inoculation with pure cultures promoted plant growth, probably because the introduced strains were physiologically active pure

cultures. Consequently they had an advantage over the indigenous population, which may have been largely inactive in soil. The results indicate that by inoculation, *Frankia* populations can be established under conditions that did not favor vesicle formation in nodules formed by the indigenous *Frankia* population. This suggests that inoculation in soils possessing a high nitrogen content might be an appropriate strategy to enhance plant growth.

Then the effect of inoculation several weeks before planting and the effect of dried alder leaves on the population structure and the N_2-fixing activity of *Frankia* in symbiosis with *A. glutinosa* were investigated. In plants the ratio of the nitrogen isotopes ^{15}N and ^{14}N was measured and the *Frankia* populations in the nodules were analyzed by *in situ* hybridization. Inoculation as well as addition of alder leaves promoted N_2-fixation and therefore plant growth. The addition of dried alder leaves into the soil resulted in an altered *Frankia* population in the nodules of non-inoculated plants. In soils without alder leaves, only group IIIa could be detected, while all nodules in soils with leaves were detectable with a probe specific for group IV. Incubation with leaves promoted a *Frankia* strain belonging to subgroup IV, which resulted in higher N_2-fixation and plant growth.

The results of this study show that *Frankia* can grow saprophytically in soils and that competition between different *Frankia* populations results in shifts of the population. This was tested by inoculating soil with *Frankia* pure cultures followed by incubation of the soil. The *Frankia* DNA content in samples taken every 1-4 weeks was determined by dot blot hybridization. Results suggested that: (i) *Frankia* can grow saprophytically, (ii) a strain belonging to group IV can grow better under the applied conditions than a strain belonging to group IIIa.

The results obtained in this thesis show that *Frankia* inoculation at or before the time of planting is an appropriate strategy to enhance N_2-fixation and plant growth even in soils with high nitrate concentrations. An alternative to inoculation could be the activation of the indigenous *Frankia* population by the addition of organic substrate, which also resulted in higher N_2-fixation and plant growth.

Zusammenfassung

Seite Leer /
Blank leaf

Zusammenfassung

Actinomyceten der Gattung *Frankia* können eine Symbiose mit sogenannten „actinorhizalen" Pflanzen eingehen, in deren Verlauf Wurzelknöllchen entstehen. In diesen Knöllchen bildet *Frankia* Vesikel, in denen molekularer Stickstoff fixiert wird. Daher können diese Pflanzen, von denen *Alnus*-Arten (Erlen) in unseren Breiten eine wichtige Rolle spielen, nicht nur für die Holzproduktion, sondern auch für Wiederaufforstungen und Verbesserung von Böden genutzt werden. Die N_2-Fixierungsrate der Symbiose wird von Faktoren wie pH, Bodenfeuchtigkeit und Temperatur beeinflusst, was man berücksichtigen muss, wenn man die Symbiose verbessern möchte.

Da *Frankia* Stämme schwer zu isolieren sind haben molekulare Methoden zunehmend an Bedeutung gewonnen, die unabhängig von Kultivierbarkeit sind. Aufgrund von Sequenzanalysen von phylogenetisch relevanten Molekülen wie der ribosomalen RNAs (rRNA) konnten die *Frankia*-Stämme, die eine Symbiose mit Erlen bilden in 5 Gruppen (I, IIIa, IIIb, IVa und IVb) unterteilt werden. Die anwachsende Menge bekannter Sequenzen führte zur Entwicklung von familien- oder gruppenspezifischen Oligosonden. Diese Sonden können in der PCR oder Ganzzell-Hybridisierung eingesetzt werden und ermöglichen das Studium der Umwelteinflüsse auf die Entwicklung der natürlich im Boden vorkommenden („endogenen") und der zugegebenen *Frankia* Populationen.

Gruppenspezifische Sonden wurden hier für eine Studie über die Durchsetzungs-fähigkeit der endogenen und der zugegebenen *Frankia* Populationen unter zwei Umweltbedingungen verwendet: (i) trockener, nitratreicher und (ii) feuchter nitratarmer Boden. Nicht-inokulierte Pflanzen enthielten entweder keine *Frankia* oder nur *Frankia* mit unförmigen Vesikeln (Gruppe IIIa). Die Blätter der Pflanzen waren chlorotisch, was darauf hinweist, dass auch der hohe Nitratgehalt im trockenen Boden keine optimalen Bedingungen für Pflanzenwachstum bot. Knöllchen von inokulierten Pflanzen dagegen enthielten *Frankia* mit Vesikeln (Gruppen IIIa und IV). Im trockenen wie im feuchten Boden wurde das Pflanzenwachstum durch die Inokulation gefördert, wohl weil die zugegebenen Stämme physiologisch aktive Reinkulturen waren, die einen Vorteil gegenüber den physiologisch inaktiven,

endogenen Kulturen hatten. Diese Ergebnisse zeigen, dass durch die Inokulation auch unter Bedingungen unter denen die Vesikelbildung durch einheimischen Stämme reduziert war, *Frankia* Populationen etabliert werden konnten.

Der Effekt der Inokulation einigen Wochen vor dem Pflanzen der Erlen sowie der Effekt von getrockneten Erlenblättern auf die Populationsstruktur und die N_2-Fixierungsrate von *Frankia* in Symbiose mit *A. glutinosa* wurden anschliessend untersucht. Dazu wurden Messungen des stabilen Stickstoffisotops ($^{15}N/^{14}N$) mit Ganzzell-Hybridisierung kombiniert. Sowohl Inokulation als auch die Zugabe von Erlenblättern förderten die N_2-Fixierung und damit das Pflanzenwachstum. Die Zugabe von getrockneten Erlenblättern führte zu einer Veränderung der *Frankia* Population in den Knöllchen von nicht-inokulierten Pflanzen: In Knöllchen von Boden ohne Erlenblätter konnte nur Gruppe IIIa, in Knöllchen von Boden mit Blättern nur Gruppe IV nachgewiesen werden. Die Zugabe von Erlenblättern förderte unter den angewendeten Bedingungen einen Stamm der Gruppe IV, was zu effizienterer N_2-Fixierung und besserem Pflanzenwachstum führte.

Die daraus folgende Annahme, dass *Frankia* saprophytisch im Boden wachsen kann wurde getestet, indem Boden mit *Frankia* inokuliert und anschliessend mehrere Wochen inkubiert wurde. Der *Frankia* DNA Gehalt wurde mit Hilfe von Dot Blot Hybridisierungen bestimmt. Die Ergebnisse zeigen, dass *Frankia* saprophytisch wachsen kann, dass es Unterschiede zwischen den Stämmen gibt und dass ein Stamm der Gruppe IV unter den angewendeten Bedingungen besser wächst.

Die Ergebnisse dieser Arbeit weisen darauf hin, dass die Zugabe von *Frankia* Reinkulturen vor und während der Pflanzzeit auch in nitratreichem Boden die N_2-Fixierungsrate und damit das Wachstum der Pflanzen stimuliert. Eine Alternative zur Inokulation ist die Aktivierung der natürlich im Boden vorkommenden *Frankia* Population, da auch das zu höherer N_2-Fixierung und besserem Pflanzenwachstum führte.

Chapter 1

General Introduction

Seite Leer /
Blank leaf

Chapter 1

General Introduction

Nitrogen-fixing actinomycetes belonging to the genus *Frankia* can form a symbiosis with the roots of so-called "actinorhizal" plants. The microorganism benefits from the symbiosis not only by receiving nutrients from the host plant but also by gaining shelter against unfavorable soil conditions [1]. The plant, on the other hand, benefits from the fixed nitrogen, especially in soils deficient in this essential element, which is one of the most limiting factors for plants in nature.

1.1. Actinorhizal plants

1.1.1. History of research on actinorhizal plants

The fact that some plants such as alders can enhance the growth rate of the surrounding vegetation or improve the soil has been known for centuries. Texts by Virgil dating from the time of the Roman Empire deal with the virtues of these plants [2]. In 1613 a poem was written about the beneficial effects of the alders on neighboring plants [3]:

"The alder, whose fat shadow nourisheth -
Each plant set neere him long flourisheth."

However, it was only in 1829 that nodule-like structures (Fig. 1.1) on the roots of alders were described for the first time. Initially they were thought to be inhabited by parasitic plants [4]. Other authors considered nodules to be examples of abnormal growth (Schacht in [5]) or to be caused by insect bites [6]. Finally, in 1860, M. Woronin could show that the inhabitant of the nodules was a microorganism which he believed to be a parasitic fungus [5].

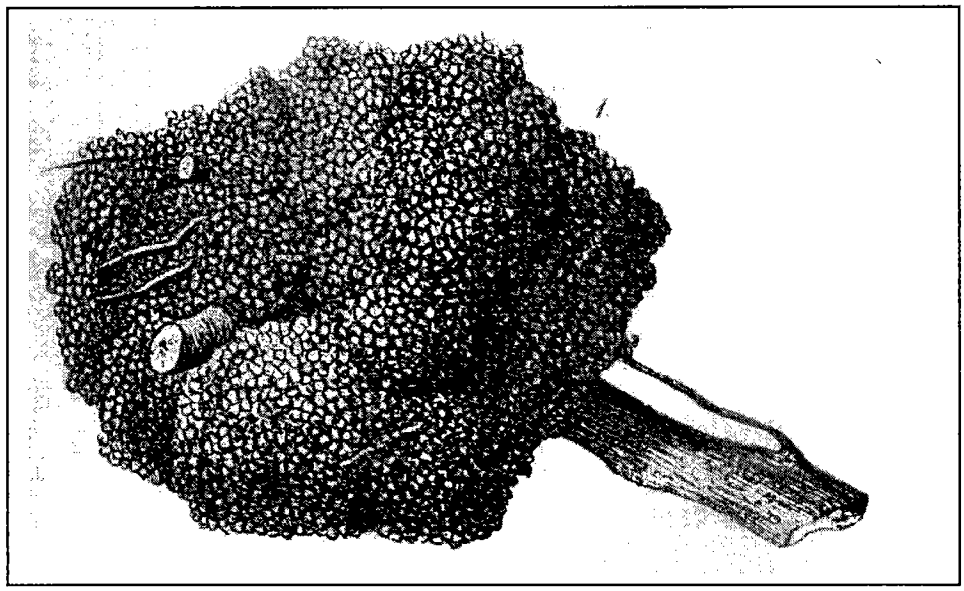


Figure 1.1. Nodule on *Alnus glutinosa* root in natural size. From [5].

Thirty years later the name *Frankia*, which is still used today, was proposed by Brunchorst in honor of his professor B. Frank [7].

Although it had been assumed that these microorganisms might not be parasites but in contrary symbionts, it was not recognized that they could fix N_2. According to Frank the plant would catch the microorganism, breed it in the protoplasma and extract the protein similar to carnivorous plants [8]:

> "Die pilzfressenden Pflanzen, um die es sich hier handelt, wissen mit noch raffinirteren Einrichtungen Pilze als ihre auserkorenen Opfer in ihr Protoplasma einzufangen, darin gross zu züchten und schliesslich zu verdauen, um so von der reichen Eiweissproduction gerade der Pilze, die die letzteren ja auch als menschliches Nahrungsmittel werthvoll macht, Nutzen zu ziehen."

The proof that *Frankia* are able to fix molecular nitrogen and therefore promote plant growth was shown only a hundred years ago [9].

In the meantime, nodules similar to those on alders had been found on several other plants belonging to different genera and families. All of these plants were known as "non-leguminous N_2-fixing plants" until 1978 when the term "actinorhizal plant" was created [10].

1.1.2. Habit and phylogeny of actinorhizal plants

Today 24 genera of plants in 8 families are known totaling almost 200 species of actinorhizal plants (Table 1.1). All of them, except *Datisca*, are perennial woody, tree-like plants or trees [11]. Otherwise, the plants are morphologically diverse including small plants such as *Dryas drummondii* as well as large trees such as *Alnus glutinosa* (Fig. 1.2) [12]. Since plants were taxonomically classified based mainly on morphological and anatomical properties [13], actinorhizal plants show great taxonomic diversity. This resulted in the opinion that actinorhizal plant families are only distantly related to one another.

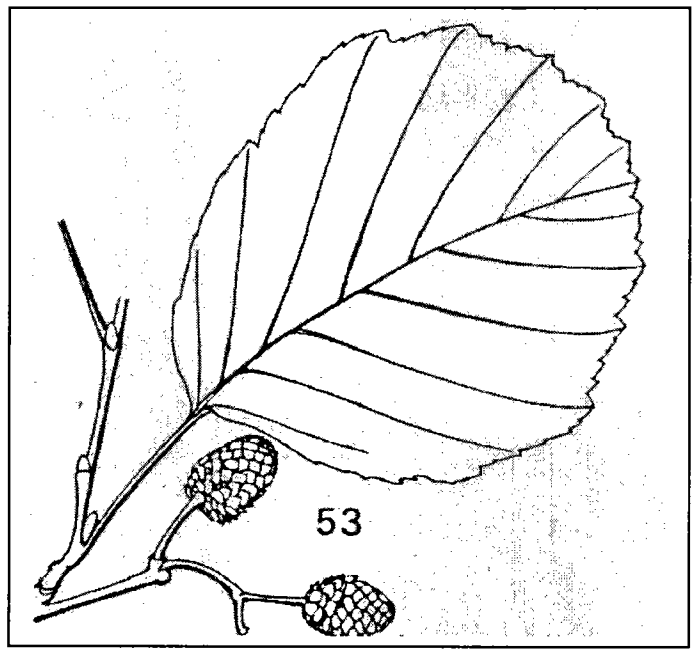


Figure 1.2. Representative figure of an *A. glutinosa* leaf and fruits from [14].

The ability to form a symbiosis with *Frankia* is not a characteristic neither of the family nor of the genus. Some of the families are completely actinorhizal (e.g. Elaeagnaceae), while others are not (e.g. Betulaceae). There are still discussions concerning the likelihood that actinorhizal plants share a common ancestor or whether the predisposition for symbiosis with N_2-fixing bacteria

such as *Frankia* evolved several times independently. Based on results of chloroplast ribulose-1,5-biphosphate carboxylase oxygenase gene (*rbcL*) sequence analysis, Soltis et al. [15] proposed a single ancestor, since all 10 families of angiosperms which are known to have members able to form N_2-fixing symbiosis occurred together in a single clade. Other families in this clade that do not form a symbiosis with *Frankia* could have either lost the ability to form such symbioses or were derived from an ancestor that provided the genetic prerequisites that later on permitted the evolution of symbiotic N_2-fixation.

In contrast in another study with a greater number of samples, four major groupings of actinorhizal species, which were interspersed with nonactinorhizal species, were resolved. The groups were not only separated by sequence differences but also by anatomical and morphological differences in nodules. Therefore, it seems likely that N_2-fixing symbioses have evolved on at least four occasions independently [16].

1.1.3. Distribution of actinorhizal plants

Actinorhizal plants are native on all continents except the Antarctic (Table 1.1). Although they can be found under different climatic conditions (arctic to subtropical), they are more common in the temperate zones. They are of great importance in high-latitude countries (Scandinavia, Canada, New Zealand), because in these areas N_2-fixing legumes are restricted by the climatic conditions [17]. Generally, actinorhizal plants can be found on sites which are deficient in nitrogen, such as wetlands, stream and lake shores, desert environments or sandy soils. Many of these environments are relatively harsh, e.g. water stress and large temperature fluctuations in the deserts or high water stress and anaerobiosis in riparian environments. Therefore actinorhizal plants have developed the ability to adapt to a variety of stresses [11].

6 genera with 10 species of actinorhizal plants are native to Europe (Table 1.2), but several other species like *Alnus rubra* or *Casuarina* have been introduced [11].

Table 1.1. Classification of actinorhizal host plant genera sensu Cronquist [13] and their geographic range. Africa (A), Eurasia (E), North America (NA), South America (SA), Oceania including Australia (O), native (n), introduced (i). Table modified from [16] and [11].

Subclass	Family	Genus	Nr of Species	A	E	NA	SA	O
Hamamelida	Betulaceae	*Alnus*	47		n	n	n	i
	Casuarinaceae	*Allocasuarina*	54					n
		Casuarina	16	i	i	i	i	n
		Ceuthostoma	2					n
		Gymnostoma	18	i	i			n
	Myricaceae	*Comptonia*	1			n		
		Myrica	28	n	n	n	n	n
Rosidae	Elaeagnaceae	*Elaeagnus*	38	i	n	n,i	i	i
		Hippophaë	2		n			
		Shepherdia	2			n		
	Rhamnaceae	*Ceanothus*	31			n		
		Colletia	4				n	
		Discaria	5				n	n
		Kentrothamnus	1				n	
		Retanilla	2				n	
		Talguenea	1				n	
		Trevoa	2				n	
	Rosaceae	*Cercocarpus*	4			n		
		Chamaebatia	1			n		
		Cowania	1			n		
		Dryas	1		n	n		
		Purshia	2			n		i
Magniliidae	Coriariaceae	*Coriaria*	5		n	n	n	n
Dilleniidae	Datiscaceae	*Datisca*	2		n	n		

Table 1.2. Geographic distribution of actinorhizal plants native to Europe. Key to countries: Balearic Isles (Bal), Belgium (B), Corsica (Cors), France (F), Spain (E), Denmark (DK), Germany (D), Czechoslovakia (CZ), Netherlands (NL), Italy (I), Russia (Ru), Greece (G), Canaries (Can), Madeira (Mad), Azores (Az); Northwest (NW); Northeast (NE); South (S). From Tutin in [18].

Family and species	**Geographical areas**	**Natural habitat**
Coriariaceae		
Coriaria myrtifolia	F, P, E, Bal, I, G	Dry woods, hedges, rocky places
Rosaceae		
Dryas octopetala	Europe (except B, NL, DK, G)	Neutral and basic soils, mountains, low altitudes in north
Datiscaceae		
Datisca cannabina	Crete	Streambanks
Myricaceae		
Myrica gale	NW Europe, extending to NW E, central D and NW Ru	Bogs, acid peatland, wet heaths
Myrica faya	E, P, Can, Mad, Az	Sandy soils, woodland, to 1500 m in Canaries
Betulaceae		
Alnus glutinosa	Most of Europe	Wet or swampy ground, riversides
Alnus incana	NE to central Europe and southern mountains	Riverside, floodplains, mountain valleys; tolerates drier soil than *A. glutinosa*
A. incana spp. *kolaensis*	Arctic Europe	
Alnus cordata	I, Cors	Neutral to basic soils, thickets, woodland
Alnus viridis	Mountains of central Europe and Balkans	Thickets
A. viridis spp. *fruticosa*	NE Ru	
A. viridis spp. *suavolens*	Cors	
Elaeagnaceae		
Elaeagnus angustifolia	Native of temperate Asia; widely naturalized from S Europe to CZ and central Ru	
Hippophaë rhamnoides	Coastal distribution, mountains of central Europe and Balkans	Sand dunes, cliffs, dry river beds and valleys in mountains

1.2. *Frankia*

1.2.1. History of research on *Frankia*

After the nodules on actinorhizal plants had been discovered, the nature of its inhabitants (Fig. 1.3) was heavily discussed. They were first thought to be parasitic plants [4], but 30 years later their anatomical structure was described by Woronin [5]. Woronin was the first to describe the nodule's content as consisting of microorganisms. He considered them to be parasitic fungi and proposed the name *Schinzia alni* because of its resemblance to fungi in the roots of other plants.

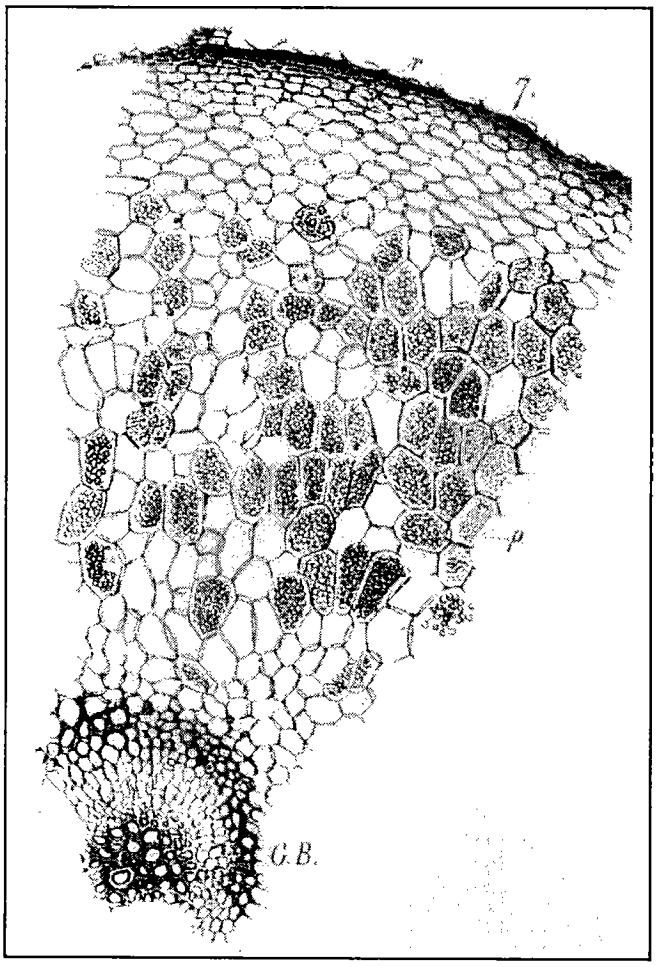


Figure 1.3. Section through a nodule of *A. glutinosa* from [5].
Vascular bundle (GB), bark (r) and parenchyma, filled with *Schinzia alni* (p).

Brunchorst [19] [7], however, realized that the microorganism in the nodules was substantially different from all other known microorganisms and, in honor of his professor B. Frank, changed the name into *Frankia subtilis.*

Finally, the microorganism in the nodules was classified based on microscopic observations as an actinomycete and placed in the family Franciaceae with one genus, *Frankia* [20].

Since these microorganisms could not be grown outside the nodule, they were thought to be obligate symbionts until 1959 when the first isolate from *Alnus glutinosa*, which was able to reinfect the host plant, was obtained by Pommer [21]. Unfortunately, this culture was lost, and until 1978 no other isolates able to reinfect the host plant could be obtained. In 1978 the strain Cp11 was isolated from *Comptonia peregrina* by Callaham [22]. In the following years hundreds of *Frankia* strains were isolated directly from nodules and grown in pure cultures. With the availability of pure cultures, research on *Frankia* increased considerably, since now it was possible to get insight into the physiology, biochemistry, genetics and taxonomy of this soil organism in culture.

1.2.2. Morphology and phylogeny of *Frankia*

The Gram positive *Frankia* grow in hyphae with a diameter from 0.5 to 2 µm and can differentiate into sporangia and vesicles (Fig. 1.4). All isolated *Frankia* strains are slow-growing, aerobic to microaerophilic microorganisms. Additionally, most *Frankia* can utilize propionate as C-source. Some strains can grow on other short chain fatty acids, glucose and other monosaccharides, as well as carboxylic acids [23]. Most isolated strains have the ability to fix molecular dinitrogen by utilizing nitrogenase located in the vesicles [24]. *Frankia* are characterized by a high G+C content, [type III] cell wall, and a phospholipid pattern of group PI [25]. Physiologically, *Frankia* strains have been classified into two groups: group A is a heterogeneous group that contains morphologically and chemically diverse strains, whereas group B is a more homogenous group containing colorless, microaerophilic strains [26]. A description of the genus *Frankia* at the species level is currently not possible.

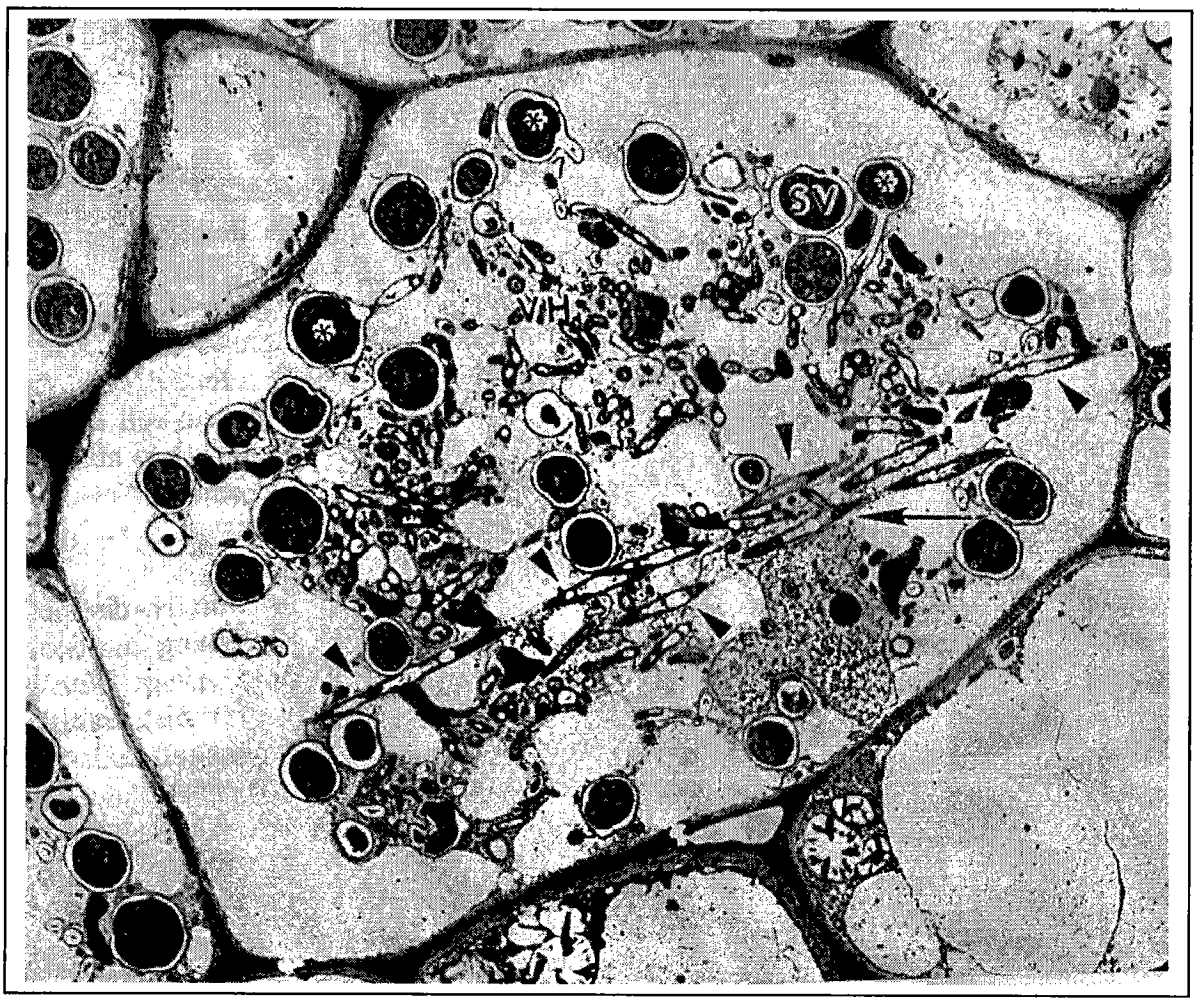


Figure 1.4. Maturing symbiotic cells in *Alnus rubra* from [27]. Three forms of *Frankia* are found: The invasive hyphal form (arrowheads) shows a linear morphology, vegetative hyphae (VH) have proliferated in the cell center after branching from the infection thread (e.g., at arrow), and symbiotic vesicles (SV) differentiate from tips of vegetative hyphae (e.g., asterisks), generally at the cell periphery. Scale bar = 10µm.

Before the first pure culture was available in 1978 [22] the first attempts to classify *Frankia* strains were made using cross-inoculation data [20]. Becking thought single *Frankia* strains to be specific for a certain plant genus or group of related genera and divided the genus *Frankia* into 10 species according to their host-plant specificity [20]. However, when pure cultures became available it could be shown that some *Frankia* strains could infect more than one plant genus [28]. Based on a study with 50 *Frankia* strains and 6 species of actinorhizal plants, Baker defined 4 host inoculation groups (Fig. 1.5) [29].

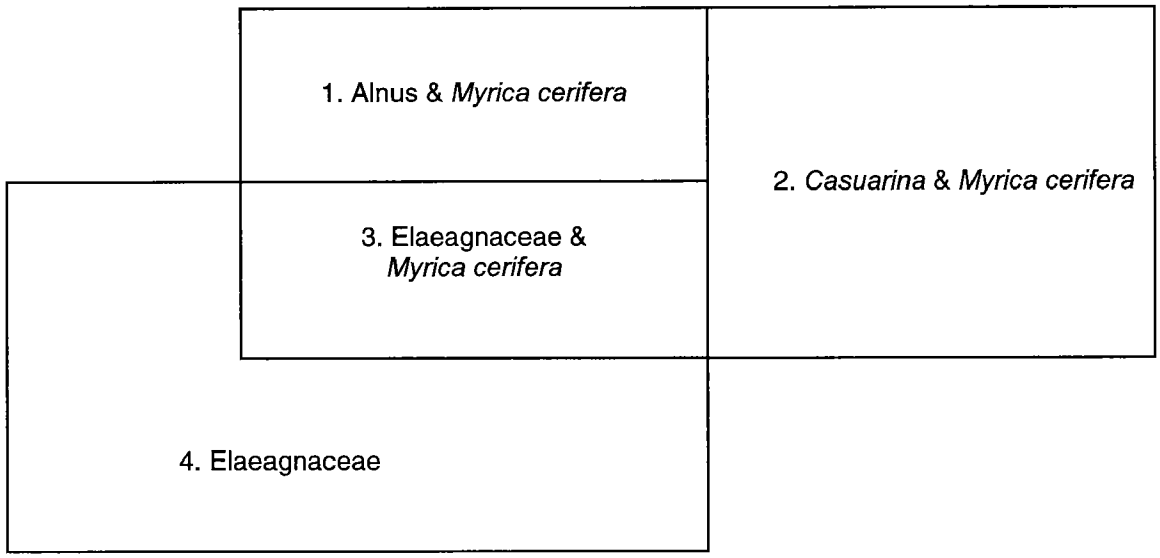


Figure 1.5. Host specificity groups according to Baker. Strains which nodulate the same host tree are grouped together [29].

It was also attempted to classify the available pure cultures according to morphological and physiological characteristics, cell chemistry and serology data [30]. Other attempts to classify the numerous *Frankia* pure cultures included the use of protein and enzyme electrophoretic patterns [31], marker sugars [32] and antigenic characteristics [33]. A major drawback to all of these methods is that pure cultures were used. Therefore the diversity of *Frankia* was underestimated as pure cultures of infective *Frankia* strains exist for only less than half of the known actinorhizal plants and the majority of *Frankia* populations in soil are not included by this approach. A better approach for *Frankia* classification involves the use of molecular techniques which are based on DNA hybridizations. One such technique is the Polymerase Chain Reaction (PCR), which makes it possible to amplify small quantities of target sequences on DNA and therefore rapidly retrieve sequence information from uncultured organisms in the environment. In recent years, molecular methods have been increasingly used to unravel phylogenetic relationships. Differences in the sequences of 16S rRNA [34] [35], 23S rRNA [36], *nif* H-D intergene [37], and *nif* H [38] have been used for the formation of groups. Such differences have also

been used for the development of group-specific primers and probes (for a detailed review see Chapter 2).

1.2.3. Occurrence of *Frankia* in different habitats

Frankia occurs in two main habitats: the soil and the nodule. For a long time *Frankia* was thought to be an obligate symbiont of the actinorhizal plants. However, the successful isolation of *Frankia* strains proved that these bacteria can be grown in pure cultures. Currently there is little information available on the physiological activities of *Frankia* in the soil, especially since only one successful isolation from soil was reported [39] and most of the information comes from *Frankia* which was trapped from soil by using host plants. It is not known whether *Frankia* can grow saprophytically (i.e. independently of a symbiotic host plant) in the soil or whether a host is necessary.

Infective *Frankia* strains can be found in at least the upper 150 cm of the soil [40]. As it would be expected for free-living organisms whose development depends on nutrient availability, the decrease in soil organic matter content is correlated with the decrease in density of *Frankia* in the deeper layers [41]. In all depths the *Frankia* population consists of various *Frankia* strains, with some strains native to the top layer, and others occurring only in layers deeper than 30 cm. This suggests that different strains possess selective advantages allowing adaption to the conditions in deeper soil layers either on the level of vegetative cells or on the level of permanent forms such as spores. [41]. Differences between strains exist. Storage in sand at high temperatures (37 °C – 40 °C) resulted in a decreased infectivity for one *Alnus* and three *Casuarina* infective strains, with greater heat-tolerance of *Casuarina* infective strains [42].

1.2.4. Can *Frankia* grow saprophytically in the soil?

The fact that many pure cultures exist and that these strains are able to utilize a wide range of carbon sources as well as fix molecular nitrogen suggests that *Frankia* might grow saprophytically. The assumption that *Frankia* can grow saprophytically in the soil is supported by the fact that *Frankia* can not only be found in soils beneath actinorhizal plants but also in soils lacking host plants

[43] [44] [45] [46]. This holds especially true for soils beneath *Betula nigra, B. pendula* and *B. pubescens*, which have an increased nodulation capacity compared to soils beneath other non-actinorhizal plants [47] [48] [49]. *Betula* is closely related to *Alnus* and might support saprophytic growth of *Frankia* by rhizosphere similarities or similar root exudates. This explains why the nodulation capacity of a soil from a *B. pendula* stand was increased after 2.5 and 5 months of storage, especially when the soil had been planted with *B. pendula* seedlings [47].

However, an increased nodulation capacity is not a proof for saprophytic growth since it could be caused by sporulation and release of spores. In addition *Frankia* hyphae can accumulate as much as 10% of their dry weight trehalose, which results in a higher desiccation tolerance [40]. Therefore not only spores but also vegetative hyphae may be able to survive some degree of desiccation in the soil. Drought-tolerance of hyphae could provide an alternative to sporulation as a means by which *Frankia* persists in dry soils, which would explain how strains unable to form spores can survive. Therefore, all *Frankia* found in soils devoid of host plants could also be spores or other permanent forms which do not grow but only survive and persist in the soil. The occurrence of only permanent forms could explain why the infection capacity of a soil originally from Jamaica did not change during over 3 years dry storage [43].

Another indication of saprophytic growth might be that *Frankia* strains are not only present in soils well outside the normal geographic range of appropriate host plants [49], but also long after host plants have disappeared from a site [43]. On the other hand the reason for this could also be a broad host range of some strains, the presence of unknown host plants, or the fact that *Frankia* strains have been transported there recently and have survived as permanent forms. It is known that *Frankia* can be transported by rivers via colloid transport. Therefore it would be possible that *Frankia* attached to small particles can also be transported by the wind [50].

Two strains inoculated into a soil via host plant roots where they were undetected prior to inoculation could be detected well outside the plot 4 years after inoculation. Although this distribution could also be explained by transport processes via water or air, saprophytic growth might be indicated by one strain

that could be detected 30 m away, while the other one was only found within 5 meters of the inoculated plot [51].

The answer to the question of whether *Frankia* can grow saprophytically or not is economically interesting since faster plant growth of actinorhizal plants as well as of the associated vegetation could be achieved by the promotion of *Frankia* growth in the soil.

1.3. Symbiosis between *Frankia* and actinorhizal plants

1.3.1. Development of the symbiosis

Nodules, which are modified lateral roots and consist of multiple lobes, develop after compatible contact between infective *Frankia* cells and host plant root. *Frankia* can infect host plant roots by two different pathways: root hair deformation or intercellular penetration.

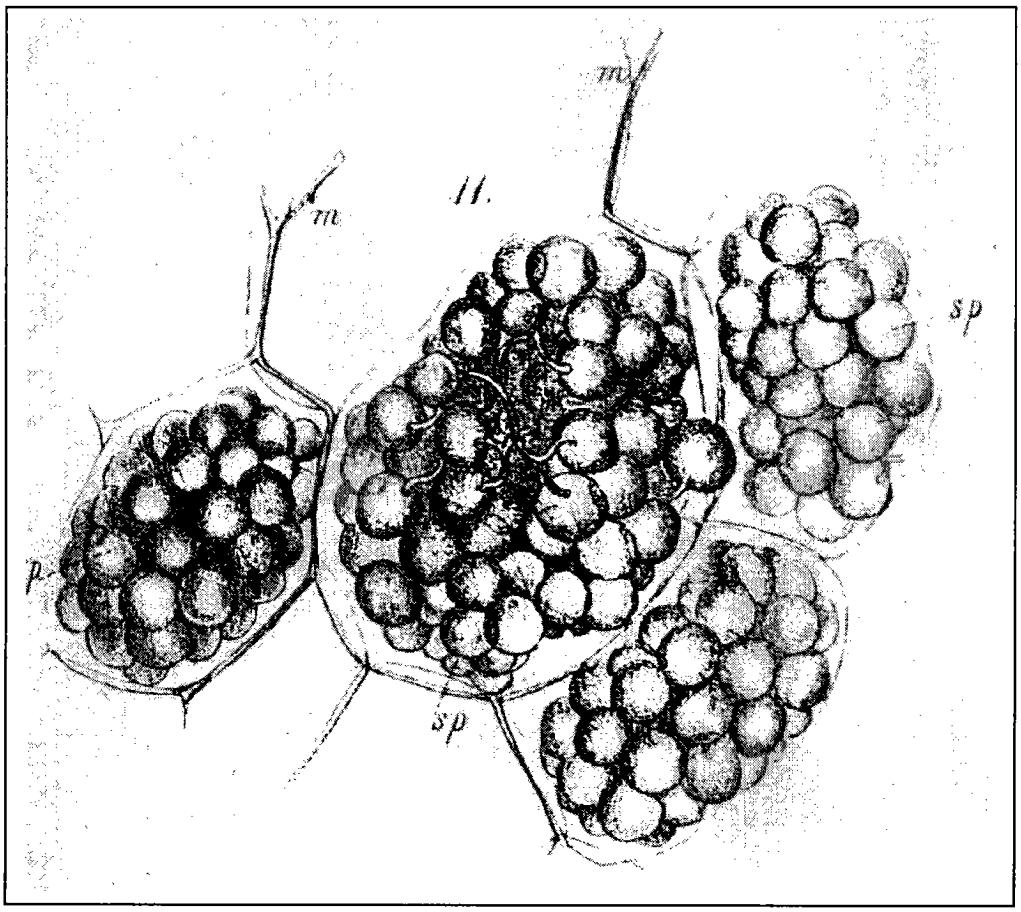


Figure 1.6. Four infected cells of a *A. glutinosa* root nodule showing *Frankia* "spores" (sp).

The "spores" are now known to be vesicles, in which N_2-fixation takes place. From [5].

The mode of infection depends on the genus of the host plant and not on the *Frankia* strain: *Alnus* is infected by root hair deformation [52] while *Elaeagnus* is infected by intercellular penetration [53]. In both cases *Frankia* penetrates the host cell walls, proliferates within the living host cells, and forms vesicles (terminal swellings that differentiate from short branch hyphae) (Fig. 1.4, 1.6).

The layered lipid envelope of the vesicles protects the nitrogenase from oxygen [54]. The fixed N_2 is released by *Frankia* in the form of ammonia and subsequently assimilated by the plant via glutamine synthetase (Fig. 1.7). In an experiment using $^{15}N_2$ as an N source, the first amino acid (glutamine) was synthesized already after 30 s. Glutamine and asparagine were the major amino acids in which the fixed N_2 was transported in the xylem [55].

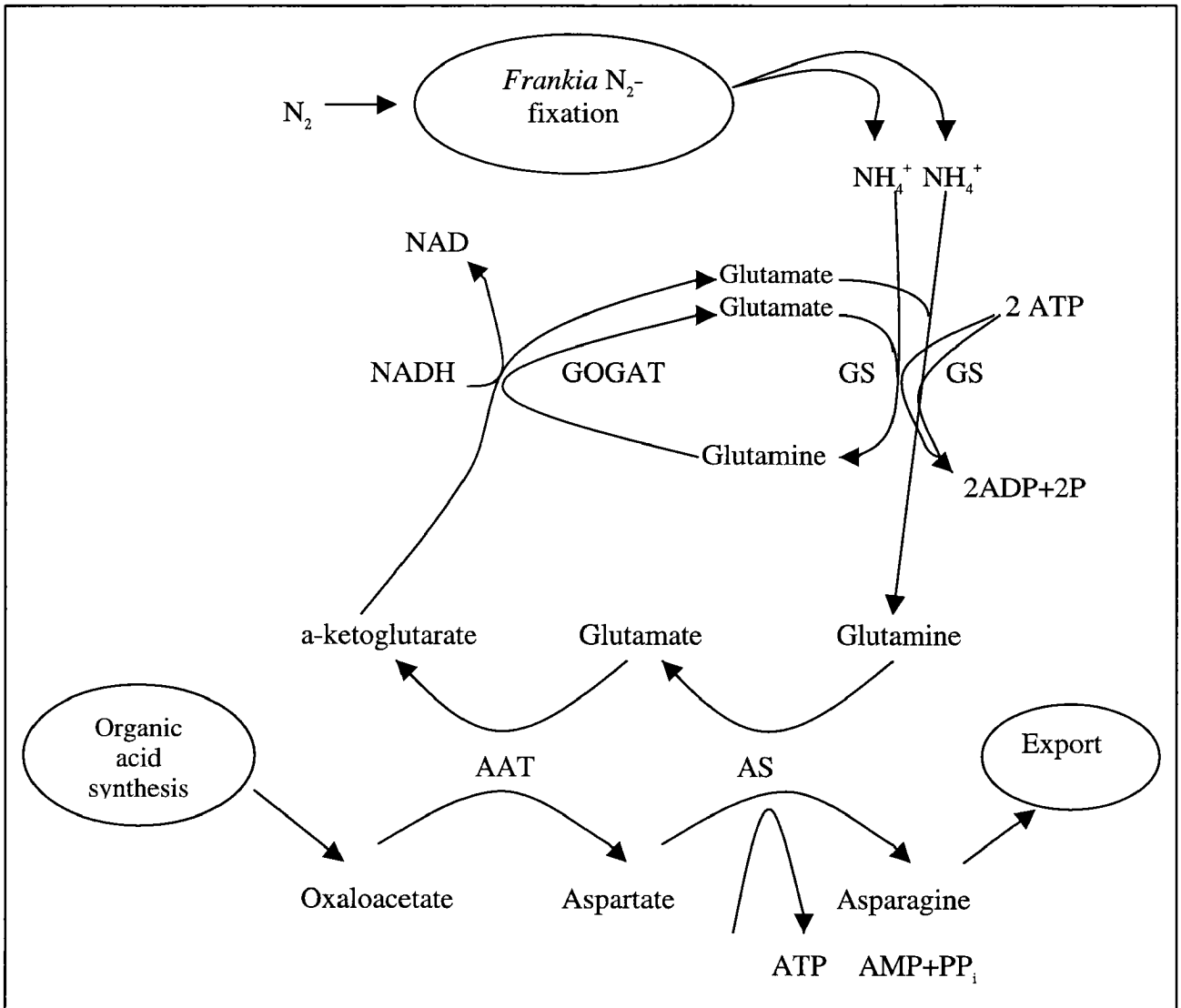


Figure 1.7. Fate of the fixed N_2 in the plant according to [55].

1.3.2. Use of actinorhizal plants

Actinorhizal plants are pioneer plants since they can extract up to 70% of their necessary nitrogen through N_2-fixation [56]. Often they are also associated with ecto- or endomycorrhiza which improve the drought resistance and uptake of nutrients such as phosphorus [57]. Therefore actinorhizal plants are able to colonize difficult habitats such as volcanic deposits, glacier moraines or eroded lands. They contribute significantly to the primary productivity of the environment, e.g. of lakes by the deposition of nitrogen rich litter [58]. Consequently, actinorhizal plants have a wide range of potential use in forestry, the most important plants being members of the Casuarinaceae family in the southern hemisphere and *Alnus* species in the northern hemisphere [59].

Alders for example can either be directly used for the production of timber or as a short rotation crop for energy production [44] [60]. In addition, the fixed N_2 is not only used by the plant but also transferred to the soil and associated vegetation [61] [62]. Therefore actinorhizal plants are also amendable for use in the improvement and exploitation of marginal or disturbed lands as well as for the enhancement of growth of the associated vegetation (Table 1.3) [63] [64] [65].

Table 1.3. Overall differences among interplanting treatments in annual height growth and estimated crown volume of 14-y-old walnut trees (*Juglans nigra*) interplanted with different trees. Values with the same letter are not significantly different. From [63].

Nurse plant	Annual height increment (m)	Crown volume (m^3)
Alnus glutinosa	0.42^a	35.94^a
Elaeagnus angustifolia	0.36^a	34.77^a
Only *Juglans nigra*	0.23^b	12.15^b

Further uses of actinorhizal plants include the planting as shade trees, erosion control and as a windbreak [66] [56].

Although the N_2-fixing potential of the actinorhizal plant species *Alnus* is high, the amount of N_2 which is actually fixed in the field can be relatively low because the efficiency of the symbiosis is often limited by unfavorable environmental conditions [59]. For use in forestry, a high N_2-fixing activity is of great importance, therefore conditions which have positive or negative effects on the symbiosis should be investigated further.

1.3.3. Influence of host and microorganism on the symbiosis

The efficiency of the symbiosis can be influenced by the host as well as by the symbiotic partner. Both partners produce substances by which the other partner is affected. For example, *A. glutinosa* seed extracts contain substances which promote growth of *Frankia* strain Arl3 [67]. These substances may be flavonoid-like compounds as in *A. rubra* seeds which have influence on the nodulation by *Frankia* either by enhancing nodulation (1 compound) or by inhibiting nodulation (2 compounds) [68]. *Frankia* on the other hand produces substances which induce root hair deformation on *A. glutinosa*, which is necessary for infection [69].

In addition the symbiosis is influenced by the genotype of both partners [70] [71]. Different clones of *A. glutinosa* inoculated with the same *Frankia* strain showed remarkable variation with respect to nodule formation, acetylene reduction activity and plant height, indicating host influence [72]. Some *Alnus* clones have a natural resistance against some ineffective *Frankia* strains but can be infected by other strains [72]. On the other hand, *A. rubra* seedlings inoculated with two different *Frankia* strains showed differences regarding plant growth, indicating influence of *Frankia* [51].

1.3.4. Influence of environmental conditions on the symbiosis

- Nitrate and ammonium: Nitrogen is a very important nutrient when focussing on N_2-fixing symbioses. For plant growth, combined N (NH_4^+, NO_3^-) is preferred over N_2 due to energetic costs of N_2-fixation, and usually results in higher plant dry matter yield. When NH_4^+ and NO_3^- are compared, NH_4^+ is

the better source of nitrogen because of the energy costs for NO_3^- reduction [73] [74]. This might be the reason why high NO_3^- or NH_4^+ concentrations in the medium to which the roots are exposed result in decreased nodulation and N_2-fixation in actinorhizal plants. Some studies identified NH_4^+ as the stronger inhibitor [75], while other authors report that NO_3^- displayed greater inhibition of nodule growth and activity than did NH_4^+ [74].

NO_3^- concentrations at which the nodule formation is completely inhibited depend on the mode of infection and are lower for host plant species infected via root hair deformation than for species infected via intercellular penetration. The reason for this is that high NO_3^- concentrations suppress root hair development [76] [77]. Concentrations at which nodule initiation is totally suppressed are between 1.5 and 3.0 mM NO_3^- for root-hair infected species [76] [78] [77], while no inhibition could be found for intercellularly infected *Eleagnus* at these concentrations [76]. The nodule formation is inhibited locally, while the nitrogenase activity in existing nodules is inhibited systemically [77].

On the other hand, the nodulation is stimulated by low N concentrations in the medium. This also results in greater biomass of the plants compared to plants grown on medium without N. In the early stages of symbiosis the combination of N_2-fixation and assimilation of NO_3^- nitrogen might therefore support optimum growth [76].

- Phosphorus: Phosphorus also exhibits some influence on nodulation and nitrogenase activity. Increased levels of phosphorus stimulated not only nodule formation and N_2-fixation [79] [80] [81] but also removed the negative effect of N on nodulation [82]. Therefore, in many agricultural and forest soils, phosphorus deficiency can be a major constraint to symbiotic N_2-fixation. This is a problem especially in highly weathered soils. Phosphorus deficiency limits symbiotic N_2-fixation not through effects on *Frankia* growth and development but through impacts on host plant growth. These impacts indirectly regulate symbiotic N_2-fixation because P requirements for optimal *Frankia* growth are equivalent to or lower than those for optimal host-plant

growth [83] [79]. N_2-fixing plants have no higher P requirement for optimal host plant growth than non-N_2-fixing plants [80].

- Cations: Cations such as Ca^{2+}, Mg^{2+}, Mn^{2+} but also Cd^{2+} and Al^{3+} influence the symbiosis between the plant and *Frankia*. Nodulation and nitrogenase activity, and therefore growth of *Casuarina equisetifolia* seedlings were weakly inhibited by $CaCl_2$, $MgCl_2$, and $MnCl_2$ and strongly inhibited by $CdCl_2$ [84]. Aluminum concentrations of 440 and 880 µM (Al monomers Al^{3+}, $Al(OH)^{2+}$ and $Al(OH)_2^{+}$) resulted in fewer nodulated plants, lower dry weights of nodules, shoots and roots, decreased N concentrations of plants, and decreased N_2-fixing activity [85]. K^+ deficiency also results in lower N_2-fixation rates [62].

  Conversely, liming of a soil with Ca^{2+} on which *A. incana* were grown resulted in better growth and higher N_2-fixation compared to plants on unlimed soils [64].

- Drought: Insufficient availability of water affects the symbiosis in two ways. First, in a dry soil, the size of the soil population of infective *Frankia* is decreased or their physiological status is changed so that they do not form nodules. In an Australian soil the number of nodules per *Casuarina* seedling decreased significantly under dry conditions [86]. The infectivity of a soil is decreased by storage under dry conditions, while it is not affected under wet conditions [42].

  Second, drought has effects directly on plants: low water potentials are accompanied by low diurnal photosynthesis and stomatal conductance of plants. This suggests that the process of symbiotic N_2-fixation, which is energetically demanding, may also be limited by the availability of photosynthetic products.

- pH: The soil pH is one of the most important variables that controls the *Frankia* soil population [87]. Nodulation and N_2-fixation activities are greatest around pH 5 to 6, but the optimal values vary for different symbioses. In soils with pH values ranging from 4.9 to 7.1 number of nodules on *A. glutinosa* was negatively correlated with the pH, whereas the number of

nodules on *E. angustifolia* was greatest at pH 6.2 [88]. For *Casuarina*, mean N concentration of nodules was significantly lower at pH 4.0 than at pH 6.0 [85]. The optimum for a *Myrica* symbiosis was at pH 5.4 [89].

- Temperature: Influences of temperature on nodulation and N_2-fixation could be caused by the different growth optima of *Frankia* strains, which are between 25°C and 37°C. Storage in sand at high temperatures (35°C - 40°C) resulted in a decreased or lost infectivity for one *Alnus* and 3 *Casuarina* infective strains, with greater heat-tolerance of *Casuarina* infective strains [42]. In addition, nitrogenase activity in *A. glutinosa* and *E. angustifolia* increased exponentially with temperatures between 10°C and 20-25°C [90].

- Other microorganisms: Bacteria such as *Pseudomonas* spp. or *Bradyrhizobium* can support nodule formation [91]. Simultaneous infection of the host plant with *Frankia* and ecto- or endomycorrhiza also have a positive effect on the symbiosis. Nodule dry weight and plant size were shown to be greater on dually-inoculated plants when compared to control plants inoculated only with *Frankia* [92]. The reason for this observation is that ecto- and endomycorrhizal plants are able to absorb more nutrients, e.g. phosphorus [93].

  However, negative effects of microorganisms on the *Frankia*-plant symbiosis are also known. Inoculation with *Azorhizobium* resulted in decreased nodule weight [91].

Regarding the great influence that the above mentioned factors have on the symbiosis, the efficiency of the symbiosis could be enhanced by optimizing the environmental conditions, which would be useful for forestry purposes.

1.3.5. Improvement of the symbiosis

The N_2-fixing symbiosis between *Frankia* and the actinorhizal plant can be improved in two ways: (i) inoculation with highly effective *Frankia* pure cultures and (ii) activation of the indigenous *Frankia* population. In the first case the strain must be a highly effective one, since not all *Frankia* isolates promote host

plant growth at the same level [51]. The most promising strains seem to be those with short generation times (< 30 hours) [94]. The strain must not only be able to infect the host plant but also to compete with the indigenous population [51] and to survive in the soil. In addition, the inoculum concentration must be optimal. The most efficient concentration of inoculum was determined to be 1-10 µl packed cell volume per plant (young seedlings) [94] or 5 µg protein of exponentially growing *Frankia* [84]. Higher concentrations seemed to have inhibitory effects.

In both cases, inoculation and activation of the indigenous population, the environmental conditions must be favorable in order to get high N_2-fixing activities. Since the "plant – *Frankia* – environment" system is an extremely complex one it is difficult to predict the effect of inoculation and of changed environmental conditions on plant growth. Various experiments must be carried out to measure the effects of differing environmental conditions. For this, different methods are available.

1.4. Methods

1.4.1. "Traditional" methods

Traditionally investigations on the morphology, physiology and phylogeny of bacteria have been carried out using pure cultures. This results in the investigation of a limited fraction of bacteria which can be cultivated and neglects the fraction which can not be grown in pure cultures. A further problem in *Frankia* research is that most of the available pure cultures were isolated from nodules, consequently only strains which were physiologically capable of infecting the host plant are available in pure cultures. In addition, the restriction to pure cultures does not allow ecological research on *Frankia* soil populations.

The presence of *Frankia* in particular soils, their relative abundance, as well as survival and persistence of *Frankia* in soil have long been studied by plant bioassays using various actinorhizal host plants "traps". Therefore, the majority of such studies have only dealt with those *Frankia* that are both compatible with the host plant and physiologically capable of infecting. There is a high probability that by this approach only a limited fraction of the total *Frankia*

population in the soil was included in the studies. In addition, as mentioned above, the nodulation capacity can be influenced by environmental conditions.

1.4.2. Molecular methods

Ecological studies on microorganisms often require the reliable identification of the microorganism without prior cultivation or in its natural habitat. Therefore molecular methods which are based on nucleic acid hybridization such as PCR, hybridization of DNA and whole cell hybridization are necessary tools.

Ecological research on *Frankia* soil populations has often been hampered by the lack of convenient and reliable methodology for their specific detection and identification in soil or nodules. In recent years, molecular techniques based on sequence analysis and DNA hybridization have been increasingly used for studying *Frankia* populations in nature. PCR-based approaches have been used to unravel the phylogenetic relationships not only of isolates but also of uncultured *Frankia* in root nodules of host plants from which no isolates have been obtained thus far. The growing database of discriminative target sequences for frankiae resulted in the development of specific primers and probes for PCR and *in situ* hybridization. With these techniques, more sophisticated studies of environmental influences on the dynamics of indigenous or introduced *Frankia* populations in plant and soil are possible. A review of molecular methods and specific probes and primers used in *Frankia* research can be found in Chapter 2.

1.4.3. Stable isotope methods

In recent years stable isotopes have been increasingly used in biological research. A great advantage of methods using stable isotopes is that these methods are sensitive and accurate [95]. For studies on N_2-fixation and plant physiology the two isotopes ^{15}N and ^{13}C are of special interest.

A very useful tool to determine the activity of N_2-fixing microorganisms is the use of the heavy isotope ^{15}N, because the natural physiological substrate for the enzyme nitrogenase can be added and its fate in the plant can be followed.

In this case the heavy isotope is added as $^{15}N_2$ and the fixation rate of the $^{15}N_2$ can be measured directly by analysis of plant material. An alternative is to add the labeled N in the form of ^{15}N-labeled nitrate or ammonium to the soil or water [96]. In this approach plants are grown in a soil containing a higher $^{15}N/^{14}N$ ratio than that of atmospheric N_2 ($^{15}N/^{14}N$ ratio = 0.3663%). Non N_2-fixing plants, which accumulate N only from the soil, are used as reference plants to determine the $^{15}N/^{14}N$ ratio of soil derived N. Consequently, the $^{15}N/^{14}N$ ratio of reference plants correspond to that of plant available N in the soil. In contrast, in plants where N is accumulated from both soil and through the N_2-fixing bacteria, the $^{15}N/^{14}N$ ratio of soil derived N in plant material is diluted through the uptake of microbiologically fixed N_2. This method is called isotope dilution method.

Experiments with *Frankia* where $^{15}N_2$ was added in the atmospheric air are usually performed by introducing the root system with nodules after the growing period in flasks containing the labeled gas mixture [41] [97]. ^{15}N-labeled nitrate or ammonium has also been used in *Frankia* research to trace the fate of the soil nitrogen compared to the fate of the nitrogen in N_2-fixing plants [74], to measure N_2-fixation and transfer of symbiotically fixed N to the associated vegetation [62].

The stable carbon isotope ratio ($\delta^{13}C$) of plant material is depleted in the heavy stable carbon isotope ^{13}C relative to that of CO_2 due to the discrimination against ^{13}C during photosynthetic pathways in plants. The $^{13}C/^{12}C$ ratio of plant material is affected by environmental conditions such as drought [98]. Therefore measurements of ^{13}C-discrimination can be used to screen for water-use efficiency [99].

1.5. Outline of the thesis

In the presented thesis the following research questions were addressed:

Chapter 2: Which molecular tools are available to follow the population dynamics of *Frankia* in soil? What primers and probes have been developed for ecological research on *Frankia* soil populations?

Chapter 3: Does inoculation with *Frankia* pure cultures promote plant growth under various environmental conditions? Are the competitive abilities of introduced *Frankia* strains similar? Can the introduced *Frankia* strains compete with the indigenous *Frankia* population?

Chapter 4: Are the introduced *Frankia* strains able to persist in the soil? Are there differences between the introduced *Frankia* strains regarding persistence and competitive abilities? Are there differences between the introduced *Frankia* strains regarding N_2-fixing activity? Is it possible to activate the indigenous *Frankia* population by adding e.g. alder leaves to the soil?

Chapter 5: Can *Frankia* strains grow saprophytically in soil (i.e. independently of a host plant)? Are there differences between the introduced *Frankia* strains?

1.6. References

1. **Miettinen, P.** 1993. The response of a free-living and an endophytic *Frankia* to extreme environmental conditions. Symbiosis 15: 121-134.

2. **Tarrant, R.F. and J.M. Trappe**. 1971. The role of *Alnus* in improving the forest environment. Plant and Soil Spec. Vol.: 335-348.

3. **Browne, W.** 1613. Britannia's Pastoral Book 1 (song 2, lines 357-358).

4. **Meyen, J.** 1829. Über das Hervorwachsen parasitischer Gebilde aus den Wurzeln anderer Pflanzen. Flora (Jena) 12: 48-64.

5. **Woronin, M.** 1866. Über die bei der Schwarzerle (*Alnus glutinosa*) und der gewöhnlichen Gartenlupine (*Lupinus mutabilis*) auftretenden Wurzelanschwellungen. Mémoires de l' Académie impériale des sciences de St. Pétersbourg 10(6): 1-13.

6. **von Jäger, G.** 1860. Über eine krankhafte Veränderung der Blüthenorgane der Weintraube. Flora (Jena) 18: 49-51.

7. **Brunchorst, J.** 1887. Die Struktur der Inhaltskörper in den Zellen einiger Wurzelanschwellungen. Bergens Mus. Aarsb. 235.

8. **Frank, B.** 1891. Über die auf Verdauung von Pilzen abzielende Symbiose der mit endotrophen Mycorhizen begabten Pflanzen, sowie der Leguminosen und Erlen. Berichte der deutschen botanischen Gesellschaft IX: 244-253.

9. **Hiltner, L.** 1895. Über die Bedeutung der Wurzelknöllchen von *Alnus glutinosa* für die Stickstoffernährung dieser Pflanze. Landwirtschaftliche Versuchsstationen 46: 153-161.

10. **Tjepkema, J.D. and J.G. Torrey**. 1979. Symbiotic nitrogen fixation in actinomycete-nodulated plants. Botanical Gazette (Chicago) Suppl. 140: i-ii.

11. **Baker, D.D. and C.R. Schwintzer**. 1990. Introduction. In: The Biology of *Frankia* and Actinorhizal Plants, Eds.: C.R. Schwintzer and J.D. Tjepkema, Academic Press, San Diego: 1-13.

12. **Moiroud, A.** 1996. Diversité et écologie des plantes actinorhiziennes. Acta Botanica Gallica 143(7): 651-661.

13. **Cronquist, A.** 1988. The Evolution and Classification of Flowering Plants. 2 ed. Allen, Lawrence, Kansas.

14. **Hess, H.E., E. Landolt and R. Hirzel.** 1984. Bestimmungschlüssel zur Flora der Schweiz. Birkhäuser Verlag, Stuttgart.

15. **Soltis, D.E., P.S. Soltis, D.R. Morgan, S.M. Swensen, B.C. Mullin, J.M. Dowd and P.G. Martin.** 1995. Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms. Proceedings of the National Academy of Sciences, USA 92: 2647-2651.

16. **Swensen, S.M. and B.C. Mullin.** 1997. Phylogenetic relationships among actinorhizal plants. The impact of molecular systematics and implications for the evolution of actinorhizal symbioses. Physiologia Plantarum 99: 565-573.

17. **Silvester, W.B.** 1977. Dinitrogen fixation by plant associations excluding legumes. In: A treatise on Dinitrogen Fixation, Eds.: R. Hardy and W. Silver, Academic Press, New York: 141-190.

18. **Wheeler, C.T. and I.M. Miller.** 1990. Current and potential uses of actinorhizal plants in Europe. In: The Biology of *Frankia* and Actinorhizal Plants, Eds.: C.R. Schwintzer and J.D. Tjepkema, Academic Press, San Diego: 365-389.

19. **Brunchorst, J.** 1886-1888. Über einige Wurzelanschwellungen, besonders diejeniegen von *Alnus* und den *Elaeagnaceen.* Untersuchungen des botanischen Instituts Tübingen 2: 150-177.

20. **Becking, J.H.** 1970. *Frankiaceae* Fam. Nov. (Actinomycetales) with one new combination and six new species of the genus *Frankia* Brunchorst 1886. International Journal of Systematic Bacteriology 20(2): 201-220.

21. **Pommer, E.H.** 1959. Beiträge zur Anatomie und Biologie der Wurzelknöllchen von *Alnus glutinosa* Gaertn. Flora (Jena) 143: 603-604.

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

23. **Akkermans, A.D.L., D. Hahn and D.D. Baker**. 1992. The family Frankiaceae. In: The Prokaryotes, Ed.: A. Balows, Springer, New York: 1069-1084.

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

25. **Benson, D.R. and W.B. Silvester**. 1993. Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. Microbiological Reviews 57(2): 293-319.

26. **Lechevalier, M.P. and H.A. Lechevalier**. 1984. Taxonomy of *Frankia*. In: Biological, biochemical and biomedical aspects of Actinomycetes, Eds.: L. Ortiz-Ortiz, L.F. Bojalil, and V. Yakoleff, Academic Press, New York: 575-582.

27. **Berg, R.H.** 1999. *Frankia* forms infection threads. Canadian Journal of Botany 77: 1327-1333.

28. **Dillon, J.T. and D.D. Baker**. 1982. Variation in nitrogenase activity among pure-cultured *Frankia* strains tested on actinorhizal plants as an indication of symbiotic compatibility. New Phytologist 92: 215-219.

29. **Baker, D.D.** 1987. Relationships among pure-cultured strains of *Frankia* based on host specificity. Physiologia Plantarum 70: 245-248.

30. **Gordon, J.C., C.T. Wheeler and D.A. Perry**. Eds. 1979. Symbiotic nitrogen fixation in the management of temperate forests, Oregon State University Press, Corvallis, Oregon: 111-123.

31. **Faure-Raynaud, M., M.A. Bonnefoy Poirier and A. Moiroud**. 1990. Diversity of *Frankia* strains isolated from actinorhizae of a single *Alnus rubra* cultivated in nursery. Symbiosis 8: 147-160.

32. **St-Laurent, L., J. Bousquet, L. Simon and M. Lalonde**. 1987. Separation of various *Frankia* strains in the *Alnus* and *Elaeagnus* host specificity groups using sugar analysis. Canadian Journal of Microbiology 33: 764-772.

33. **Baker, D., W.L. Pengelly and J.G. Torrey**. 1981. Immunochemical analysis of relationships among isolated Frankiae (*Actinomycetales*) International Journal of Systematic and Bacteriology 31: 148-151.

34. **Nazaret, S., B. Cournoyer, P. Normand and P. Simonet**. 1991. Phylogenetic relationships among *Frankia* genomic species determined by use of amplified 16S rDNA sequences. Journal of Bacteriology 173(13): 4072-4078.

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

36. **Hönerlage, W., D. Hahn, K. Zepp, J. Zeyer and P. Normand**. 1994. A hypervariable 23S rRNA region provides a discriminating target for specific characterization of uncultured and cultured *Frankia*. Systematic and Applied Microbiology 17: 433-443.

37. **Cournoyer, B., M. Gouy and P. Normand**. 1993. Molecular phylogeny of the symbiotic actinomycetes of the genus *Frankia* matches host-plant infection process. Molecular Biology and Evolution 10(6): 1303-1316.

38. **Normand, P. and J. Bousquet**. 1989. Phylogeny of nitrogenase sequences in *Frankia* and other nitrogen-fixing microorganisms. Journal of Molecular Evolution 29: 436-447.

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

40. **Burleigh, S.H. and J.O. Dawson**. 1994. Dessication tolerance and trehalose production in *Frankia* hyphae. Soil Biology and Biochemistry 26: 593-598.

41. **Nalin, R., P. Normand and A.-M. Domenach**. 1997. Distribution and N_2-fixing activity of *Frankia*-strains in relation to soil depth. Physiologia Plantarum 99: 732-738.

42. **Sayed, W.F., C.T. Wheeler, H.H. Zahran and A.A.M. Shoreit**. 1997. Effect of temperature and soil moisture on the survival and symbiotic effectivness of *Frankia* ssp. Biology and Fertility of Soils 25: 349-353.

43. **Zimpfer, J.F., C.A. Smyth and J.O. Dawson**. 1997. The capacity of Jamaican mine spoils, agricultural and forest soils to nodulate *Myrica cerifera*, *Leucaena leucocephala* and *Casuarina cunninghamiana*. Physiologia Plantarum 99: 664-672.

44. **Arveby, A.S. and K. Huss-Danell**. 1988. Presence and dispersal of infective *Frankia* in peat and meadow soils in Sweden. Biology and Fertility of Soils 6(1): 38-44.

45. **Maunuksela, L., K. Zepp, T. Koivula, J. Zeyer, K. Haahtela and D. Hahn**. 1999. Analysis of *Frankia* in three soils devoid of actinorhizal plants. FEMS Microbiology Ecology 28: 11-21.

46. **Huss-Danell, K. and A.-K. Frej**. 1986. Distribution of *Frankia* in soil from forest and afforestation sites in northern Sweden. Plant and Soil 90: 407-418.

47. **Smolander, A. and M.L. Sarsa**. 1990. *Frankia* strains of soils under *Betula pendula*: Behaviour in soil and pure culture. Plant and Soil 122: 129-136.

48. **Smolander, A.** 1990. *Frankia* populations in soils under different tree species - with special emphasis on soil under *Betula pendula*. Plant and Soil 121: 1-10.

49. **Paschke, M.W. and J.O. Dawson**. 1992. The occurrence of *Frankia* in tropical forest soils of Costa Rica. Plant and Soil 142: 63-67.

50. **Huss-Danell, K., D. Uliassi and I. Renberg**. 1997. River and Lake sediments as sources of infective *Frankia* (*Alnus*). Plant and Soil 197: 35-39.

51. **McEwan, N.R., E.M.O. Gould, E. Lumini, J.D. McNeill and C.T. Wheeler**. 1998. Competitivity and persistence of *Frankia* in a managed environment. In: Nitrogen fixation with non-legumes. Proceedings of the 7th international symposium on nitrogen fixation with non-legumes, Eds.: A. Malik, M.S. Mirza, and J.K. Ladha, Kluwer Academic Publishers, Dordrecht: 315-326.

52. **Berry, A.M., L. McIntyre and M.E. McCully**. 1986. Fine structure of root hair infection leading to nodulation in the *Frankia* - *Alnus* symbiosis. Canadian Journal of Botany 64: 292-305.

53. **Miller, I.M. and D.D. Baker**. 1985. The initiation, development and structure of root nodules in *Elaeagnus angustifolia*. Protoplasma 128: 107-119.

54. **Alskog, G. and K. Huss-Danell**. 1997. Superoxide dismutase, catalase and nitrogenase activities of symbiotic *Frankia* (*Alnus incana*) in response to different oxygen tensions. Physiologia Plantarum 99: 286-292.

55. **Baker, A. and R. Parsons**. 1997. Rapid assimilation of recently fixed N2 in root nodules of *Myrica gale*. Physiologia Plantarum 99: 640-647.

56. **Dommergues, Y.R.** 1996. La fixation d'azote chez les plantes actinorhiziennes et ses applications. Acta botanica Gallica 143(7): 663-679.

57. **Sempavalan, J., C.T. Wheeler and J.E. Hooker**. 1995. Lack of competition between *Frankia* and *Glomus* for infection and colonization of roots of *Casuarina equisetifolia* (L.). New Phytologist 130: 429-436.

58. **Goldman, C.R.** 1961. The contribution of Alder trees (*Alnus tenuifolia*) to the primary productivity of Castle lake, California. Ecology 42(2): 282-288.

59. **Dommergues, Y.R.** 1996. Contribution of actinorhizal plants to tropical soil productivity and rehabilitation. Soil Biology and Biochemistry 29(5/6): 931-941.

60. **Eckholm, E.** 1980. Introduction. In: Firewood crops, National Academy of Sciences, Washington, D.C.: 1-9.

61. **Huss-Danell, K. and H. Ohlsson.** 1992. Distribution of biomass and nitrogen among plant parts and soil nitrogen in a young *Alnus incana* stand. Canadian Journal of Botany 70: 1545-1549.

62. **Ekblad, A. and K. Huss-Danell.** 1995. Nitrogen fixation by *Alnus incana* and nitrogen transfer from *A. incana* to *Pinus sylvestris* influenced by macronutrients and ectomycorrhiza. New Phytologist 131: 453-459.

63. **Dawson, J.O. and J.W. Van Sambeck.** 1993. Interplanting woody nurse crops promotes differential growth of black walnut saplings. In: Proceedings, 9th Central Hardwood Forest Conference, Eds.: A.R. Gillespie, P.E. Pope, G. Rink, U.S. Department of Agriculture, Forest Service, North Central Forest Experiment Station, West Lafayette, IN: 455-463.

64. **Huss-Danell, K.** 1986. Growth and production of leaf litter nitrogen by *Alnus incana* in response to liming and fertilization on degenerated forest soil. Canadian Journal of Forest Research 16: 847-853.

65. **Mikola, P.** 1975. Afforestation of bogs after industrial exploitation of peat. Silva Fenn 9: 101-115.

66. **MacDicken, K.G. and N.T. Vergara**, eds. 1990. Agroforestry: Classification and Management. Wiley, New York.

67. **Ringø, E., E. Clausen, E. Løvaas, M. Van Ghelue and B. Solheim.** 1995. Effects of extracts of *Alnus glutinosa* seeds on growth of *Frankia* strain Arl3 under static and fermentor culture conditions. Plant and Soil 176: 283-288.

68. **Benoit, L.F. and A.M. Berry.** 1997. Flavonoid-like compounds from seeds of red alder (*Alnus rubra*) influence host nodulation by *Frankia* (Actinomycetales). Physiologia Plantarum 99: 588-593.

69. **Van Ghelue, M., E. Løvaas, E. Ringø and B. Solheim.** 1997. Early interactions between *Alnus glutinosa* and *Frankia* strain Arl3. Production

and specificity of root hair deformation factor(s). Physiologia Plantarum 99: 579-587.

70. **Simon, L., A. Stein, S. Coté and M. Lalonde**. 1985. Performance of in vitro propagated *Alnus glutinosa* (L.) Gaertn. clones inoculated with *Frankiae*. Plant and Soil 87: 125-133.

71. **Hall, R.B., H.S. McNabb, C.A. Maynard and T.L. Green**. 1979. Toward development of optimal *Alnus glutinosa* symbiosis. Botanical Gazette 140 (Suppl.): S120-126.

72. **Hahn, D., M.J.C. Starrenburg and A.D.L. Akkermans**. 1988. Variable compatibility of cloned *Alnus glutinosa* ecotypes against ineffective *Frankia* strains. Plant and Soil 107: 233-243.

73. **Troelstra, S.R., R. Wagenaar and W. Smant**. 1992. Growth of actinorhizal plants as influenced by the form of N with special reference to *Myrica gale* and *Alnus incana*. Journal of Experimental Botany 43(255): 1349-1359.

74. **Baker, A., G.F. Hill and R. Parsons**. 1997. Alteration of N nutrition in *Myrica gale* induces changes in nodule growth, nodule activity and amino acid composition. Physiologia Plantarum 99: 632-639.

75. **Zhang, Z. and J.G. Torrey**. 1985. Biological and cultural characteristics of the effective *Frankia* strain HFPCCcl3 (Actinomycetales) from *Casuarina cunninghamiana* (Casuarinaceae). Annals of Botany 56: 367-378.

76. **Kohls, S.J. and D.D. Baker**. 1989. Effects of substrate nitrate concentration on symbiotic nodule formation in actinorhizal plants. Plant and Soil 118: 171-179.

77. **Arnone, J.A., S.J. Kohls and D.D. Baker**. 1994. Nitrate effects on nodulation and nitrogenase activity of actinorhizal *Casuarina* studied in split-root systems. Soil Biology and Biochemistry 26(5): 599-606.

78. **Thomas, K.A. and A.M. Berry**. 1989. Effects of continuous nitrogen application and nitrogen preconditioning on nodulation and growth of *Ceanothus griseus* var *horizontalis*. Plant and Soil 118: 181-187.

79. **Yang, Y.** 1995. The effect of phosphorus on nodule formation and function in the *Casuarina-Frankia* symbiosis. Plant and Soil 176: 161-169.

80. **Redell, P., Y. Yun and W.A. Shipton**. 1997. Do *Casuarina cunninghamiana* seedlings dependent on symbiotic N_2 fixation have higher phosphorus requirements than those supplied with adequate fertilizer nitrogen? Plant and Soil 189: 213-219.

81. **Sanginga, N., S.K.A. Danso and G.D. Bowen**. 1989. Nodulation and growth response of *Allocasuarina* and *Casuarina* species to phosphorus fertilization. Plant and Soil 118: 125-132.

82. **Huss-Danell, K.** 1997. Actinorhizal symbioses and their N_2 fixation. New Phytologist 136: 375-405.

83. **Yang, Y., W.A. Shipton and P. Redell**. 1997. Effects of phosphorus supply on in vitro growth and phosphatase activity of *Frankia* isolates from *Casuarina*. Plant and Soil 189: 75-79.

84. **Selim, S. and J. Schwencke**. 1995. Simple and reproducible nodulation test for *Casuarina*-compatible *Frankia* strains: Inhibition of nodulation and plant performance by some cations. Arid Soil Research and Rehabilitation 9: 25-37.

85. **Igual, J.M., C. Rodriguez-Barrueco and E. Cervantes**. 1997. The effects of aluminum on nodulation and symbiotic nitrogen fixation in *Casuarina cunninghamiana* Miq. Plant and Soil 190: 41-46.

86. **Dawson, J.O., D.G. Kowalski and P.J. Dart**. 1989. Variation with soil depth, topographic position and host species in the capacity of soils from an Australian locale to nodulate *Casuarina* and *Allocasuarina* seedlings. Plant and Soil 118: 1-11.

87. **Smolander, A., C. Van Dijk and V. Sundman**. 1988. Survival of *Frankia* strains introduced into soil. Plant and Soil 106: 65-72.

88. **Zitzer, S.F. and J.O. Dawson**. 1992. Soil properties and actinorhizal vegetation influence nodulation of *Alnus glutinosa* and *Elaeagnus angustifolia* by *Frankia*. Plant and Soil 140: 197-204.

89. **Bond, G.** 1951. The fixation of nitrogen associated with the root nodules of *Myrica gale* L., with special reference to its pH relation and ecological significance. Annals of Botany 15(60): 447-460.

90. **Zitzer, S.F. and J.O. Dawson**. 1989. Seasonal changes in nodular nitrogenase activity of *Alnus glutinosa* and *Elaeagnus angustifolia*. Tree Physiology 5: 185-194.

91. **Girgis, M.G., Y. Ishac, H. El-Lakany and Y. Dommergues**. 1990. Enhancing nodulation and growth of *Casuarina glauca* planted in substrate already infected with *Frankia*. In: Fifth International Symposium on Nitrogen Fixation with Non-Legumes, Florence, Italy, Kluwer Academic Publishers.

92. **Chatarpaul, L., P. Chakravarty and P. Subramaniam**. 1989. Studies in tetrapartite symbiosis. Plant and Soil 118: 145-150.

93. **Russo, R.O.** 1989. Evaluating alder-endophyte (*Alnus acuminata-Frankia*-Mycorrhizae) interactions. Plant and Soil 118: 151-155.

94. **Steele, D.B., K. Ramirez and M.D. Stowers**. 1989. Host plant growth response to inoculation with *Frankia*. Plant and Soil 118: 139-143.

95. **Danso, S.K.A.** 1995. Assessment of biological nitrogen fixation. Fertilizer Research 42(1/3): 33-41.

96. **Fried, M. and V. Middelboe**. 1977. Measurement of amount of nitrogen fixed by a legume crop (Soybean). Plant and Soil 47(3): 713-715.

97. **Kurdali, F., G. Rinaudo, A. Moiroud and A.M. Domenach**. 1990. Competition for nodulation and $^{15}N_2$-fixation between a Sp^+ and a Sp^- *Frankia* strain in *Alnus incana*. Soil Biology and Biochemistry 22: 57-64.

98. **Farquhar, G.D., K.T. Hubick, A.G. Condon and R.A. Richards**. 1989. Carbon isotope fractionation and plant water-use efficiency. In: Stable isotopes in ecological research, Eds.: P.W. Rundel, J.R. Ehleringer, and K.A. Nagy, Springer, New York.

99. **Hubick, K.T., G.D. Farquhar and R. Shorter**. 1986. Correlation between water-use efficiency and carbon isotope discrimination in

diverse peanut (Arachis) germplasm. Australian Journal of Plant Physiology 13(6): 803-816.

Chapter 2

Assessing *Frankia* populations in plants and soil using molecular methods

D. Hahn, A. Nickel, J. Dawson

FEMS Microbiology Ecology 29 (1999), 215-227

Seite Leer /
Blank leaf

Chapter 2

Assessing *Frankia* populations in plants and soil using molecular methods

2.1. Abstract

In recent years molecular approaches have increasingly supplemented nodulation-dependent detection methods for studying *Frankia* populations in nature. The new methods are revealing much about the genetic diversity and distribution of *Frankia*, as well as refining and expanding knowledge about endophyte-host specificities. PCR-based approaches have been used to unravel the phylogenetic relationships of isolates, as well as of uncultured endophytes in root nodules of many actinorhizal plants from which no isolates have been obtained. Comparative sequence analysis of PCR-amplified 16S rDNA led to the emendation of the family Frankiaceae to contain only the genus *Frankia* with four main subdivisions: i) a large group mainly comprising *Frankia alni* and other typical N_2-fixing strains belonging to the *Alnus* and the *Casuarina* host infection groups, respectively; ii) uncultured endophytes of *Dryas*, *Coriaria*, and *Datisca* species; iii) strains of the *Elaeagnus* host infection group; and iv) atypical non-N_2-fixing strains. Considerable diversity among both cultured *Frankia* strains and uncultured endophytes in nodules was indicated using RFLP analyses of PCR-amplified fragments of the 16S rRNA gene, the glutamine synthetase II (*gln*II) gene, the intergenic spacer (IGS) of the 16S-23S rRNA operon, or the IGS between the nitrogenase *nif*H and *nif*D (*nif*H-D) or the *nif*D and *nif*K (*nif*D-K) gene. The growing database of discriminative target sequences for frankiae is increasingly exploited for studies on the distribution of specific *Frankia* populations in the environment using PCR or *in situ* hybridization. Until recently, most studies have focused on the analysis of *Frankia* populations in root nodules, the natural locale of enrichment for this organism. These populations, however, represent only the fraction of physiologically active, infecting frankiae in soils rather than the total *Frankia*

population. Future approaches to studies of *Frankia* populations should therefore incorporate the many opportunities for more than just phylogenetic analyses, the description of diversity and studies of *Frankia* populations in nodules. The molecular approaches open the door to more sophisticated studies of environmental influences on the dynamics of indigenous or introduced *Frankia* populations in plants and soil. These studies may lead to advancements in the management of actinorhizal plants and *Frankia*, provided specific *Frankia* populations can be attributed with silviculturally beneficial features. Such features include persistence and growth in soil, competition with less efficient *Frankia* populations for nodule formation, prompt and efficient nodule formation, and ultimately superior N_2-fixing capacity.

2.2. Introduction

Members of the actinomycetous genus *Frankia* are generally characterized as N_2-fixing organisms that form root nodules in symbiosis with more than 200 species of non-leguminous woody plants in 25 genera of angiosperms [1]. *Frankia* populations inhabit two distinct ecological niches, the root nodule and the soil. Studies on *Frankia* populations in both niches, however, have been impeded by problems encountered in the isolation and identification of frankiae [1]. *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 20 years and hundreds of isolates are available. Most of the isolates can be classified into four host infection groups, i.e. those nodulating i) *Alnus* and *Myrica*, ii) *Casuarina* and *Myrica*, iii) *Myrica* and *Elaeagnus*, and iv) members of the Elaeagnaceae (*Elaeagnus*, *Hippophaë*, *Shepherdia*) [2]. This classification, however, is incomplete because isolates from several actinorhizal plant taxa are still lacking. Furthermore, some isolates cross the boundaries between host infection groups [3] and, in addition to the diazotrophic, infective (typical) frankiae, some non-N_2-fixing, non-infective isolates (atypical frankiae) have been obtained from actinorhizal nodules [4]. All *Frankia* strains belong to the spore (-) type, whereas isolates producing spores in root nodules (spore (+) type) have not yet been obtained in pure culture. Even for *Frankia* strains of the

spore(-)-type, no general isolation protocols have been developed and consequently only a small percentage of isolation attempts succeed.

While a considerable amount of information is available on *Frankia* strains isolated from root nodules and on their interaction with their host plants (see [5], [6] for review), data on *Frankia* populations in soil are scarce. 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 [7]. 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 [8]. Using these methods, nodulation units between 0 and 4600 g^{-1} soil have been obtained for different soils [9]. The nodulation capacity of soil from birch-stands, for example, appears to be as high or higher as that of soil from alder-stands [10]. These results demonstrate that members of the genus *Frankia* can survive and remain infective in soils that are devoid of host plants [10] [11].

Apart from the need for large numbers of test plants, the use of this bioassay is hampered by its selectivity, since only nodule-forming *Frankia* populations can be detected. Questions on *Frankia* populations belonging to host infection groups other than the test plants [2], or on non-nodulating *Frankia* populations of the same host infection group [4] are neglected. Other drawbacks of the bioassay include the failure to analyze specific *Frankia* populations, the inability to quantify competition for infection between *Frankia* populations in a sample, and variable compatibilities of host plants to *Frankia* populations [8]. 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* [8]. Furthermore, the correlation of the nodulation unit, which can theoretically be induced by a single spore, a hyphal fragment or a colony, with cell numbers remains problematic [9].

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. Such an *in situ* analysis is unaffected by the limitations of culturability because sequences on DNA or rRNA rather than microorganisms are targeted. The feasibility of molecular approaches for the *in situ* analysis of *Frankia* populations, however, is most influenced by the target organism and the heterogeneity of the habitat. The analysis is more difficult in highly heterogeneous habitats such as pristine forest soils which contain a high diversity of small numbers of physiologically active and inactive bacterial populations (5000 to 6000 genomes per g soil [dry wt]). Consequently, the majority of studies on *Frankia* populations have been focused on pure cultures and uncultured endophytes in nodules which comprise monoxenic enrichments of one physiologically active bacterial population.

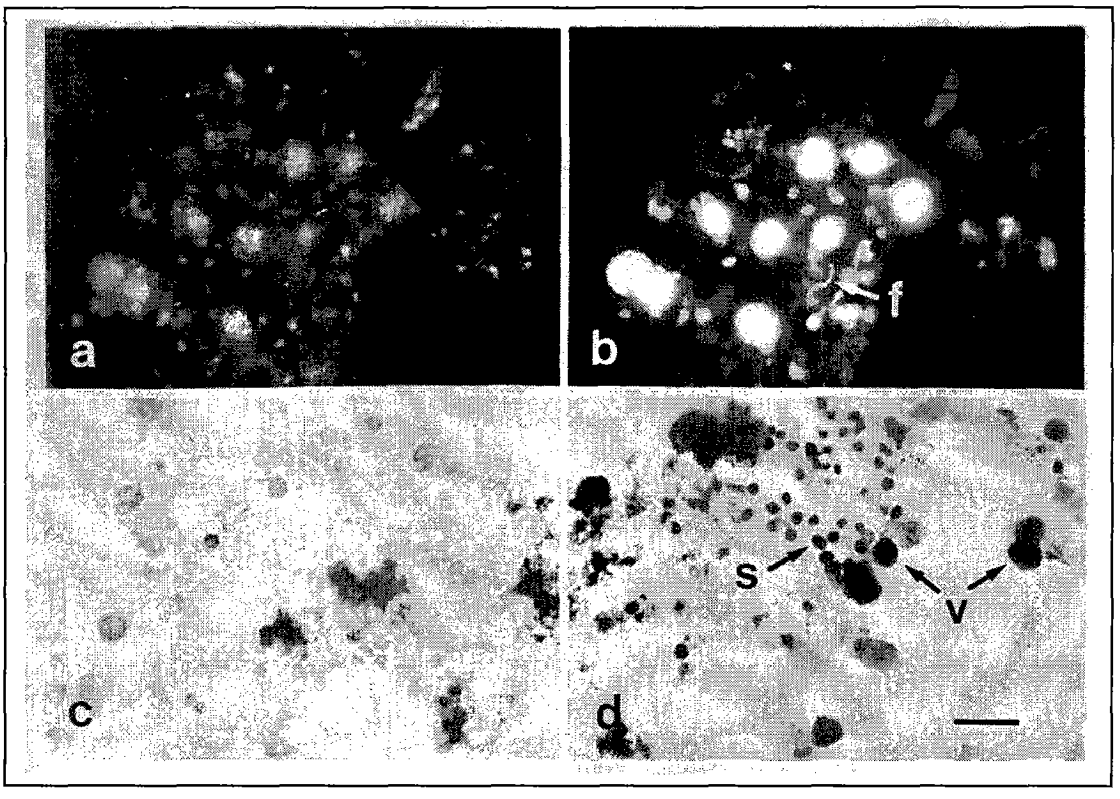


Figure 2.1. *In situ* detection of three cell types of *Frankia* (filaments (f); vesicles (v); spores (s)) in nodule homogenates after hybridization with fluorescent (Cy3-labeled) (b, epifluorescence micrograph: filterset HQ-Cy3; AHF Analysentechnik, Tübingen, Germany; G 535/50, FT 565, BP 610/75) or digoxigenin-labeled (d, brightfield micrograph) oligonucleotide probes. Figure a shows the epifluorescence micrograph corresponding to figure b after DAPI staining (filter set Zeiss 02; G 365, FT 395, LP 420). Figure c shows a brightfield micrograph of the background staining after hybridization with a non-binding, digoxigenin-labeled probe. Bar represents 5 µm.

The most prominent advancement in studies on the ecology of *Frankia* has resulted from the use of the polymerase chain reaction (PCR) which enabled researchers to amplify minute quantities of target sequences on DNA and to rapidly retrieve sequence information from uncultured organisms in the environment. The potential of this technique was initially exploited for the phylogenetic analysis of isolates and uncultured endophytes in nodules but has also been used for the detection and analysis of specific *Frankia* populations in nodules and soil as specific sequence information became available (Table 2.1).

Table 2.1. Primer combinations for PCR-based analyses of frankiae at different levels of resolution

Target	Primer	Sequence (5' - >3')	Target organism	Reference
16S rDNA	FGPS958	CTTGACATGCAGGGAAATC	Frankiaceae	[12]
	FGPS1093'	GCAACATAGGACGAGGGTTG		
	FGPS56-352	TGCAASTCGAGCGUGGUGCTT	Genus *Frankia*	[13]
	FGPS1509'-153	AAGGAGGGGATCCAGCCGCA		
	FGPS59	AAGTCGAGCGGGGAGCTT	Genus *Frankia*	[14]
	FGPS305'	CCAGTGTGGCCGGTCGCCCTCTCAG		
	1115	AGGGTTGCGCTCGTTG	*Genus* Frankia	[15]
	PCRII	CTGGTGGTGTGGAAAG		
	FGPS849	GCCTTGGGAGTACGGCCGCA	*Genus* Frankia	[16]
	FGPS1146'	GGGGCATGATGACTTGACGTC		
	FGPS989ac	GGGGTCCGTAAGGGTC	*Frankia* strains of the *Alnus* and *Casuarina* host infection groups	[3]
	FGPS1176'	GGGGCATGATGACTTGACGTC		
	FGPS989e	GGGGTCCTTAGGGGCT	*Frankia* strains of the *Elaeagnus* host infection group	[3]
	FGPS1176'	GGGGCATGATGACTTGACGTC		
	FGPS154p	ATGCCGGATATGACGCTA	*Frankia* of the spore (+) type in nodules of *A. incana*	[17]
	FGPS305'	CCAGTGTGGCCGGTCGCCCTCTC		
	FGPS59	AAGTCGAGCGAGGGGCTT	*Frankia* of the spore (-) type in nodules of *A. incana*	[17]
	FGPS188m'	CACCAGATGCCCGGAGGT		
	FGPS940	CGAAGAACCTTACCAA	Endophyte in nodules of *Coriaria* spp.	[18]
	FGPS1098'	CGCTGGCAACATAGA		

Target	Primer	Sequence (5' - >3')	Target organism	Reference
23S rDNA	23InsV	MADGCGTAGNCGAWGG	Frankiaceae	[19]
	23Fra	CCATCATCCGTACGCTA		
	23InsVFra	CAGGCGTAGTCGATGG	Genus *Frankia*	[20]
	23Fra	CCATCATCCGTACGCTA		
	FraV	GATAACGGGTTGATATTCC	Genus *Frankia*	[11]
	23Fra	CCATCATCCGTACGCTA		
	FraV	GATAACGGGTTGATATTCC	*Frankia* of the *Alnus* host infection group I (non-nitrogen-fixing strains)*	[11]
	23B1.9	GAGGGCGACTAAGGGAA		
	AFAr	ACCAGGTTCAGCATGGGC	*Frankia* of the *Alnus* host infection subgroups IIIa,b*	[11], [20]
	23Arl3	CCAGACACATCTCCGAAAAG		
	AFMr	GTCCATGCTGAGGCCAGG	*Frankia* of the *Alnus* host infection subgroup IVa*	[11], [20]
	23Mut(II)	CCACACACACCCCCTAA		
	AFMr	GTCCATGCTGAGGCCAGG	*Frankia* of the *Alnus* host infection subgroup IVb*	[11]
	23AvN	GGAAAGCCCCCACACCCCCA		
	AFAr	ACCAGGTTCAGCATGGGC	*Frankia* of the *Alnus* host infection subgroups IIIa,b*	[11]
	23AvC	AAGCCTCTACACAGACC		
IGS 16S-23S rDNA	FGPS1493	GGCTGGATCACCTCCTTTCT	Genus *Frankia*	[12]
	FGPL2054'	CCGGGTTTCCCCATTCGG		
IGS *nif*H-D	FGPH750	GAAGACGATCCCGACCCCGA	Genus *Frankia*	[12]
	FGPD913'	GGTCGGGACCTCATCCTCGA		
	FGPH256	GAGTCCGGTGGCCCGGAGCC	Genus *Frankia*	[21]
	FGPD913'	GGTCGGGACCTCATCCTCGA		
IGS *nif*D-K	FGPD807-85	CACTGCTACCGGTCGATGAA	Genus *Frankia*	[22]
	FGPK700'	CGAGGTAGGTCTCGAAACCGG		
	FGPD807-85	CACTGCTACCGGTCGATGAA	Genus *Frankia*	[23]
	FGPK333'-355	CCGGGCGAAGTGGCT		
***gln*II**	FGgs19	TACATC TGGATCCACGGCAC	Genus *Frankia*	[24]
	FGgs417'	GCCGACGCCGCAGTAGTA		
REP	REP1R-I	IIIICGICGICATCIGGC	All organisms	[25], 36],
	REP2-1	ICGICTTATCIGGCCTAC		[26]

Target	Primer	Sequence (5' - >3')	Target organism	Reference
ERIC	ERIC1R	ATGTAAGCTCCTTGGGGATTCAC	All organisms	[25], [26]
	ERIC2	AAGTAAGTGACTGGGGTGAGCG		
BOX	BOXA1R	CTACGGCAAGGCGACGCTGACG	All organisms	[11], [26]
AP	A-01	CAGGCCCTTC	All organisms	[27]
	A-05	AGGGGTCTTG	All organisms	[27]

M = (A/C); W = (A/T); S = (G/C); V = (A/G/C); D = (A/G/T); N = (A/G/C/T); I = Inosin; U = Uracil;
*according to the classification of Hönerlage et al. [19].

Further studies have been based on hybridization assays such as the *in situ* hybridization technique which focused on the microscopic detection of labeled probes hybridized to target sequences in fixed cells of frankiae at different taxonomic levels (Table 2.2, Fig. 2.1). This method avoids the *in vitro* amplification of the target sequences because naturally amplified molecules like ribosomal RNAs are used as targets. Up to date, however, it has only been used to analyze *Frankia* populations in root nodules.

Table 2.2. Probes for hybridization-based analyses of frankiae at different levels of resolution

Target	Probe	Sequence (5' - >3')	Target Organism	Reference
16S rRNA	FP	ATAAATCTTTCCACACCACCAG	Genus *Frankia*	[28]
	FGPS962-280	ACATGCASRGAAATC	Genus *Frankia*	[13]
	FGPS59	AAGTCGAGCGGGGAGCTT	Genus *Frankia*	[14]
	FGPS305	CCAGTGTGGCCGGTCGCCCTCTCAG	Genus *Frankia*	[14]
	EFP	GCAGGACCCTTACGGATCCC	Non-nitrogen-fixing *Frankia* strains from the *Alnus* host infection group	[29]

Target	Probe	Sequence (5' - >3')	Target Organism	Reference
16S rRNA	IFP	ACGGGACCCTTTAAAGGACCCA	Non-nitrogen-fixing *Frankia* strains from the *Alnus* host infection group	[29]
	16Sp(+)	AGATGCCCGGTAGCGTC	*Frankia* in spore (+) type nodules of alders	[17]
	16Sp(-)	AGATGCCCGGAGGTGTC	*Frankia* in spore (-) type nodules of alders	[17]
	COR/DAT	GAGCCCTTACGGACCCCGTATC	*Frankia* in nodules of *Coriaria* and *Datisca*	[30]
	Dc2	GCGGGACCCCTAAGG	*Frankia* strain Dc2	[30]
23S rRNA	23Fra	ATCGCATGCCTACTACC	Genus *Frankia*	[31]
	AFA	ACCAGGTTCAGCATGGGC	*Frankia* of the *Casuarina* (group II) and *Alnus* host infection group (IIIa,b)*	[19], [31]
	AFM	CCTGGCCTCAGCATGGAC	*Frankia* of the *Alnus* host infection group IV*	[19], [31]
	23B1.9	AAGGGAATCAGCGGGAG	*Frankia* of the *Alnus* host infection group I (non-nitrogen-fixing strains)*	[31]
	23Ccl3	CCGAACACACCTCCGAAGAG	*Frankia* of the *Casuarina* host infection group (group II)*	[31]
	23Arl3	CCAGACACATCTCCGAAAAG	*Frankia* of the *Alnus* host infection group IIIa*	[31], [20]
	23Ves	TACTACCAGCCGGGATC	*Frankia* of the *Alnus* host infection group IIIa*	[31]

Target	Probe	Sequence (5' - >3')	Target Organism	Reference
23S rRNA	23Mid	GATCCCACCCTCCCAGAC	*Frankia* of the *Alnus* host infection group IIIa*	[31]
	23B32(II)	ACACACACATCTCCGAA	*Frankia* of the *Alnus* host infection group IIIb*	[11], [31], [20]
	23AvC	AAGCCTCTACACAGACC	*Frankia* of the *Alnus* host infection group III*	[11]
	23Mut(II)	CCACACACACCCCCTAA	*Frankia* of the *Alnus* host infection group IVa*	[11], [31], [20]
	23AvN	ACCCCCACACCCCCGAAAGG	*Frankia* of the *Alnus* host infection group IVb*	[11], [31]
	23Ea1.12	CTCGAAGGCAGAACAAGA	*Frankia* of the *Elaeagnus* host infection group (group VI)*	[31]
	23KG4	ACACACATATCTTCGAAAAG	*Frankia* strain AgKG'84/4	[31]
	23ARg	ACATGCACATCCCCGAAAGG	*Frankia* strain ARgP5	[31]
***nif* H**	PN1	AGCCGCAAGACCGAC	*Frankia* strain Arl3	[32]
	PN2	AGCCGTAACACCGAC	*Frankia* strain ACoN24d	[32]
	PN1'	CGGTCGGTCTTGCGGCTG	*Frankia* strain Arl3	[33]
	PN2'	CGGTCGGTGTTACGGCTG	*Frankia* strain ACoN24d	[33]
Plasmid pFQ56	pFQ121	pFQ56 cloned into vector pBR322; nick translated	pFQ56-bearing *Frankia* strains (ACoN24d)	[34]

S = (G/C); R = (A/G); * according to the classification of Hönerlage et al. [19]

2.3. Phylogeny of isolates and uncultured endophytes in nodules

The assignment of isolates or uncultured nodule endophytes to the genus *Frankia* is usually based on PCR-assisted sequence retrieval from isolated DNA, subsequent cloning and sequencing of the amplicons, and finally a comparative analysis of sequences derived from pure cultures or uncultured populations with those of existing reference collections. Generally, genes with a high phylogenetic significance such as ribosomal RNAs (rRNAs) have been targeted for such analysis. A differentiation of the genus *Frankia* from other N_2-fixing organisms has also been achieved by using the size of the intergenic spacer (IGS) between the nitrogenase *nif*H and *nif*D (*nif*H-D) genes [12] or the *nif*D and *nif*K (*nif*D-K) genes [22]. Frankiae have also been differentiated from other actinomycetes by using the glutamine synthetase II (*gln*II) gene as the target for PCR [24].

Comparative sequence analysis of PCR-amplified 16S rDNA of pure cultures of *Frankia*, as well as of uncultured endophytes in root nodules, led to the emendation of the family Frankiaceae to contain only the genus *Frankia* [35]. Taxa related to the genus *Frankia* were the genera *Geodermatophilus*, "*Blastococcus*", *Sporichthya*, *Acidothermus*, and *Actinoplanes* (2.2). The initial separation of closely related strains belonging to the *Alnus* or *Casuarina* host infection group from strains of the *Elaeagnus* host infection group [16] was superceded by the proposal that there are four main subdivisions within the genus *Frankia*: i) a large group mainly comprising *Frankia alni* and other typical N_2-fixing strains belonging to the *Alnus* and the *Casuarina* host infection groups, respectively; ii) uncultured endophytes of *Dryas*, *Coriaria*, and *Datisca* species; iii) strains of the *Elaeagnus* host infection group; and iv) atypical non-N_2-fixing strains (Fig. 2.2) [35].

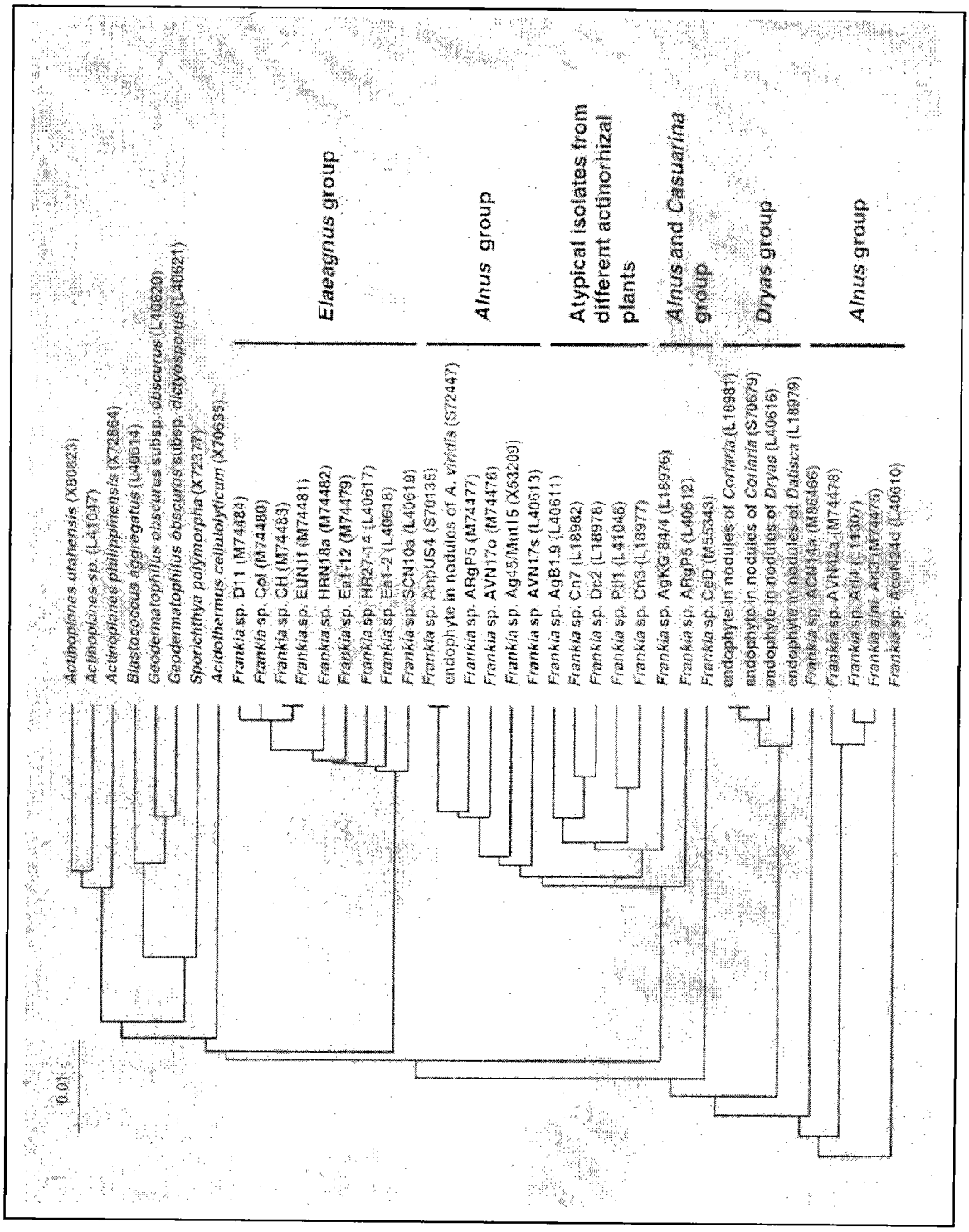


Figure 2.2. A neighbor joining tree of selected members of the genus *Frankia* (approximately 30% of all sequences available to date) and closely related organisms derived from the EMBL/GenBank database using FASTA through GCG package. A phylogenetic tree was calculated after alignment of sequences using the CLUSTAL W program. The distance scale indicates the expected number of changes per sequence position.

This differentiation was largely confirmed by comparative sequence analysis of an actinomycetes-specific 23S rRNA insertion of 35 *Frankia* strains [19]. *Frankia* strains belonging to the *Casuarina* and *Elaeagnus* host infection groups were characterized by no or only small sequence variation, whereas strains belonging to the *Alnus* host infection group could be separated into four subgroups, three containing typical N_2-fixing strains and a fourth one containing only non- N_2-fixing strains [19]. Recently, a further subgroup containing typical N_2-fixing *Frankia* strains of the *Alnus* host infection group was proposed [11]. Two other non-infective and non-N_2-fixing strains isolated from *Coriaria nepalensis* and *Purshia tridentata*, respectively, were assigned to the group containing non-N_2-fixing *Frankia* strains. These results confirmed earlier studies based on 16S rRNA sequence analysis which revealed a close relationship of non-N_2-fixing and non-nodulating isolates obtained from nodules of *Datisca cannabina* [36] and of *C. nepalensis* [36] to the genus *Frankia*.

PCR-based approaches were extensively used to investigate the phylogenetic relationships of uncultured endophytes in root nodules of many actinorhizal plants from which isolates have not been obtained. Since nodules comprise monoxenic enrichments of one organism, uncultured endophytes in nodules can be analyzed using less than 1 mg (fresh wt.) of nodule tissue, single lobes of nodules or fewer than 10 vesicle clusters as the starting material for template preparation [25] [37]. Analyses of 16S rDNA revealed that the uncultured endophytes in root nodules of *Coriaria arborea* and *Coriaria plumosa* were identical to one another [37], and that the endophytes in nodules of *Coriaria* and *Datisca* were highly related. Both comprised a separate lineage within the genus *Frankia*, along with the endophyte in nodules of *Dryas drummondii* (*Dryas* group) (Fig. 2.2) [18]. The isolates obtained from nodules of *D. cannabina* and *C. nepalensis* were not related to this group [35]. Further studies also assigned the endophyte in nodules of *Ceanothus americanus* to this group [38], and also the uncultured endophytes in nodules of *Ceanothus griseus* and *Purshia tridentata* which had identical sequences to those obtained from nodules of *D. drummondii* [37] .

The analysis of 16S rDNA from uncultured endophytes in root nodules of several *Ceanothus* spp. suggests that the endophytes in *Ceanothus* nodules

share a common ancestor to that of frankiae infecting members of the Elaeagnaceae [25]. A close relationship to members of the *Elaeagnus* host infection group was also demonstrated for isolates and/or uncultured endophytes of nodules from *Gymnostoma* [39] and from *Colletia hystrix*, *Talguenea quinquenervia*, *Trevoa trinervis*, *Retanilla ephedra*, *Discaria serratifolia*, and *D. toumatou* [37]. Similar to the endophyte in nodules of *C. americanus*, the uncultured endophyte in nodules of *C. caerulens* was assigned to the *Dryas* group [40]. Non-N_2-fixing and non-nodulating isolates from nodules of *C. caerulens*, however, clustered to the atypical frankiae [40]. Finally, the phylogenetic position of uncultured non-N_2-fixing endophytes in nodules of *Alnus glutinosa* was found to be different from that of non-N_2-fixing isolates derived from *A. glutinosa* nodules from the same geographic location. These nodule endophytes belonged to a hitherto undescribed cluster within the genus *Frankia* [41].

2.4. Diversity of isolates and *Frankia* in nodules

Studies on diversity of *Frankia* have most often focused on isolates and uncultured endophytes in nodules. Using rRNA as the target for dot blot hybridization, nodules of about 1 mg (fresh wt.) provided sufficient target molecules for reliable detection of the *Frankia* strain [42]. *In situ* hybridization allowed for the analysis of *Frankia* populations in nodules of *Alnus glutinosa*, *A. incana*, *A. viridis*, and *A. nepalensis* on the subgroup-level with a detection limit of one cell. The analysis revealed the presence of only one *Frankia* population in every nodule homogenate [31]. Filaments and vesicles in nodules of the spore (-) type as well as filaments, vesicles and spores in nodules of the spore (+) type always belonged to the same group although specific *Frankia* populations did not correlate with nodules of either type. *In situ* hybridization also showed that isolates obtained from nodules of *A. incana*, used as the capture plant after inoculation with soil from a birch-, pine- or spruce-stand, respectively, accounted for a significant portion of the *Frankia* population in root nodules [11]. However, only isolates obtained from nodules on capture plants inoculated with soil from the birch-stand seemed to represent the total *Frankia*

population in root nodules. Nodules induced after inoculation with soil from the pine- or spruce-stand also inhabited *Frankia* populations which had not been isolated and which could not be identified [11].

Discrimination of frankiae at a lower taxonomic level has often been based on restriction fragment length polymorphism (RFLP) analysis. RFLP analysis on DNA extracted from individual nodule lobes on *Ceanothus* and subsequent hybridization with two random *Frankia* probes or a *nif*D-H gene probe revealed a considerable diversity among *Frankia* strains symbiotic with *Ceanothus* [43]. Differences in RFLP patterns were observed among plants at a single geographic site and between geographical sites but no correlation between RFLP pattern and geographic site was detected. Today, RFLP analyses are usually performed on PCR amplified nucleic acids such as the 16S rDNA [44], the *gln*II gene [24], the IGS of the 16S-23S rRNA operon [21], the IGS of *nif*H-D [24] or the IGS of *nif*D-K [22]. RFLP analysis of PCR amplified 16S rDNA fragments from both cultured *Frankia* and uncultured endophytes in nodules, for example, have permitted discrimination among different *Frankia* genomic groups [44]. However, the discriminative ability of this technique on 16S rDNA amplicons depends on the restriction enzyme used and usually requires a comparative analysis of patterns generated with different restriction enzymes [13].

In contrast, RFLP analysis of PCR amplified *nif*D-K IGS regions with the endonuclease *Hae*III has already resulted in the generation of seven different profiles when uncultured *Frankia* populations in nodules on *Elaeagnus angustifolia* L. were analyzed [45]. RFLP analysis of PCR amplified *nif*D-K IGS regions resulted in the clustering of 22 *Frankia* strains of the *Elaeagnus* host infection group into seven genomic groups with three closely related RFLP clusters for six *Frankia* strains infective on both *Alnus* and *Elaeagnus* [46]. In combination with DNA-DNA hybridization, the analysis indicated that the ability of these strains to nodulate *Alnus* and *Elaeagnus* was a monophyletic trait shared by three genomic species [46].

A combination of RFLP analysis of 16S-23S rDNA IGS amplicons digested with the restriction enzyme *Nci*I with hybridization to specific oligonucleotide probes of PCR amplified *nif*H-D revealed a close relationship between 60 *Frankia*

isolates obtained from root nodules of *Casuarina equisetifolia* and reference strain CeD [21]. In contrast to these studies which suggest a small genetic diversity among *Frankia* strains that infect members of the family Casuarinaceae, five distinct genetic groups were recognized using a set of different restriction enzymes when amplicons of the 16S-23S rDNA IGS and the *nif*D-K IGS of pure cultures of *Frankia* belonging to the *Casuarina* host infection group and uncultured populations in nodules on two *Casuarina* and two *Allocasuarina* species were analyzed [47]. Again, all pure cultures had similar patterns and were assigned to a group along with most of the uncultured endophytes in nodules from *C. equisetifolia*. A second group consisted of two uncultured endophytes from *C. equisetifolia*, whereas three further groups were comprised only of uncultured endophytes from *C. cunninghamiana*, *A. torulosa*, and *A. littoralis* [47].

At the sub-species level, arbitrary primers [27], as well as intervening sequences of *Frankia* genomic DNA amplified by PCR with oligonucleotide primers targeting consensus motifs of repetitive elements common to prokaryotic genomes such as REP (repetitive extragenic palindromic) or ERIC (enterobacterial repetitive intergenic consensus) elements (rep-PCR) [11], [26], provided an effective means to distinguish closely related strains. Genomic fingerprints on DNA of root nodules of several *Ceanothus* spp. using rep-PCR differentiated twelve distinct groups, indicating considerable genetic diversity of *Frankia* in the nodules sampled [25]. A similar high degree of diversity was obtained for isolates from nodules of *A. incana* which could not be differentiated by comparative sequence analysis of a 23S rRNA insertion but which all exhibited different rep-PCR patterns [11].

The database available on discriminative target sequences for *Frankia* populations permits rapid analysis of uncultured *Frankia* populations in nodules. Specific *Frankia* populations in nodules were analyzed by probing PCR products with specific oligonucleotide probes [12], [32] [32] [30]. Based on this approach, for example, the distribution pattern of spore (-) and spore (+) populations of *Frankia* in an alder stand was investigated [17]. Uncultured *Frankia* populations were characterized in root nodules of *Alnus glutinosa* by probing PCR amplicons of a 23S rRNA insertion [19] [31] and by *in situ*

hybridization revealing the presence of only one *Frankia* population in root nodules of different alders from different locations [31]. Hybridization assays also make possible the analysis of distribution profiles of plasmid-containing and plasmid-free *Frankia* populations in nodules [34] as well as the competitive ability of specific *Frankia* strains to form nodules [33] [20].

2.5. *Frankia* in soil

Many studies on the occurrence of frankiae in soil have focused on the infecting *Frankia* population using a combination of plant bioassays and the subsequent analysis of *Frankia* in nodules formed. The occurrence of *Frankia* populations in soil samples collected from different localities in Pakistan, for example, was analyzed by inoculating *Coriaria nepalensis* and *Datisca cannabina* plants [30]. Hybridization with specific 16S rDNA targeted probes as well as the analysis of partial, PCR amplified 16S rDNA sequences of uncultured endophytes in nodules revealed four types of *Frankia* sequences and one non-*Frankia* sequence. Another example showed the presence of *Frankia* populations infective on *Elaeagnus angustifolia* L. in different depths of the same soil collected at a site devoid of Elaeagnaceae [45]. RFLP analysis of PCR amplified *nif*D-K IGS from uncultured endophytes in root nodules of the capture plants resulted in seven different profiles within the infecting *Frankia* community. The diversity was maintained throughout the soil column although the relative distribution of profiles as well as the number of nodules varied. A decrease in density of *Frankia* strains was correlated with a decline of soil organic matter content with soil depth [45].

First attempts to quantify *Frankia* populations directly in soil dealt with 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 10^4cells [28]. This detection limit was comparable with that obtained by PCR with 10^4 genomic units g^{-1} soil [48]. A genomic unit was defined as the amount of frankiae containing a single genome, though frankiae appeared to contain two rDNA operons. The detection limit could be increased to 10 genomic units g^{-1} soil by optimizing

PCR conditions and using booster PCR [48]. Today, quantification of *Frankia* populations usually employs PCR-MPN using nested [48] or booster PCR [14]. 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 2940 nodulation units g^{-1} soil, was just a small portion of the total population of *Frankia* as measured by genomic units, which ranged from 2000 to 92000 genomic units g^{-1} soil [48]. These results suggested that the nodulation capacity of a soil was controlled largely by the physiological status or host compatibility of the *Frankia* populations rather than by the total population size [48].

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 [20]. Comparable results were obtained in a combined approach with plant bioassay and PCR-assisted detection of *Frankia* populations directly in soil when *Frankia* populations in three soils devoid of actinorhizal plants were studied [11]. 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 [11]. 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 abundances of the target sequences in the original sample. Therefore, the size of the *Frankia* populations detected may differ significantly. Furthermore, the analysis only focused on a small number of target groups and might therefore be incomplete. This assumption was supported by the discovery of *Frankia* populations in nodules of the capture plants after inoculation with soil from the pine- and spruce-stands which could not be identified [11]. Comparative sequence analysis of *Frankia* populations in these nodules may therefore lead to the detection of other *Frankia* populations or also result in the discovery of new *Frankia* populations.

Future approaches to studies on uncultured *Frankia* populations should incorporate the many opportunities for more than just phylogenetic analyses and the description of diversity of uncultured endophytes in nodules. Methods for ecophysiological studies of uncultured *Frankia* populations in soil are available and can be used in microcosm studies with defined *Frankia* populations (Fig. 2.3). Studies on natural populations in the environment, however, rely on the accurate analysis of diversity of uncultured *Frankia* populations in the soil and rhizosphere. Studies should be extended to closely related organisms from nodules or from other habitats. From nodules of *Casuarina equisetifolia*, for example, N_2-fixing actinomycetes were isolated which formed a new clade and did not belong to the genus *Frankia* [49]. Similar results were obtained for isolates of rock dwelling bacteria which were shown to be equidistantly related to *Geodermatophilus obscurus*, *Frankia alni*, *Sporichthya polymorpha*, and *Acidothermus cellulolyticus* [50]. Other studies could retrieve sequence information from bacteria on ancient wall paintings identifying members or close relatives of the genera *Halomonas*, *Clostridium*, and *Frankia.* In the rhizosphere of *Alnus viridis*, PCR-assisted 16S rDNA sequence retrieval with *Frankia*-specific primers, resulted in the detection of several sequences typical for bacteria with a low or high DNA G+C content [13]. Only two sequences appeared to be in the same line of descent as *Frankia* and *Geodermatophilus* representing yet uncharacterized soil bacteria.

For the study of uncultured *Frankia* populations in soil, PCR-based techniques using specific primers could help to obtain sequence information. A major obstacle to this approach, however, is that the initial PCR is based on a primer set designed on sequence information usually obtained from a few cultured and/or uncultured frankiae which may lead to the use of genus-specific primers that do not bind to groups of uncultured *Frankia* populations, resulting in a significant underestimation of the diversity of the genus. The design of specific or generic primers must be refined constantly to take into account new sequences in DNA data banks [13]. These approaches open the door to more

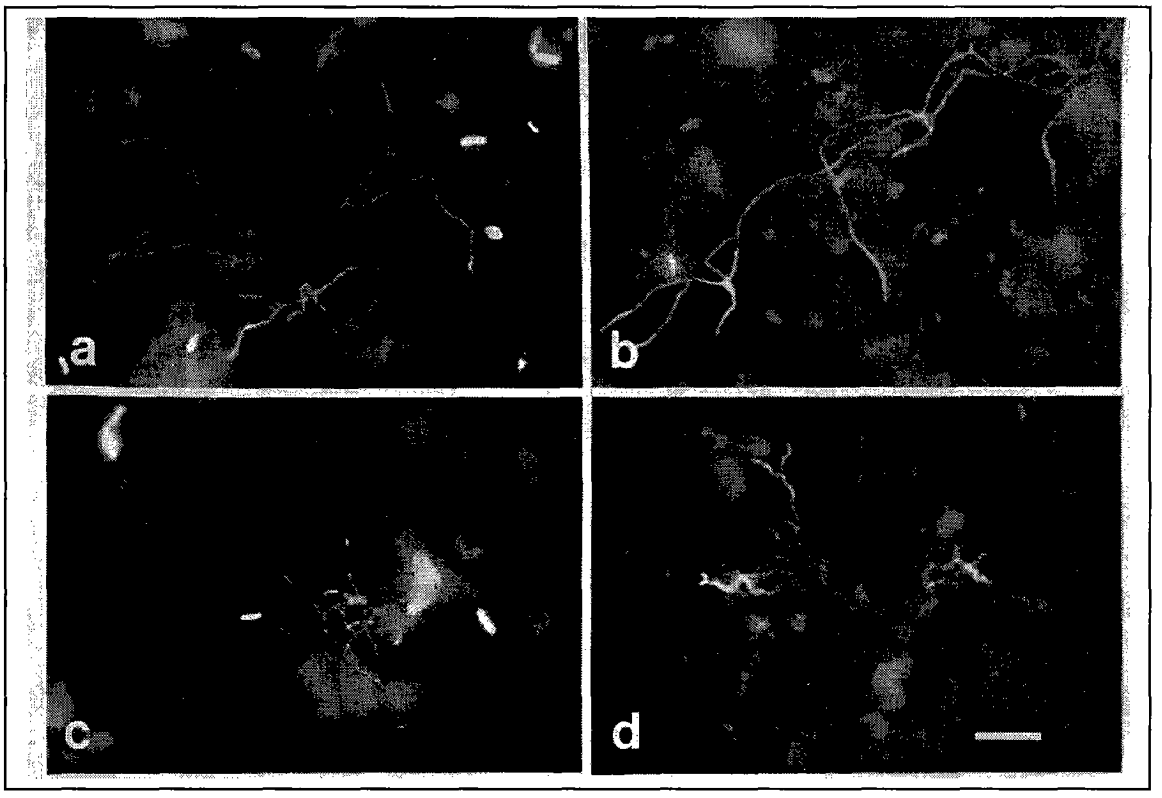


Fig. 2.3. *In situ* detection of *Frankia* strain AgB1.9 in soil incubated at 20°C for one (a, b) and eight (c, d) weeks. After hybridization with Cy3-labeled oligonucleotide probe EUB338 targeting all members of the domain Bacteria (a, c) both rods and filamentous bacteria are detected. Hybridization with the AgB1.9-specific probe B1.9 (b, d) shows filamentous bacteria only. Bar represents 5 µm.

sophisticated studies of environmental factors such as soil pH, soil matric potential, the quality of organic material, or the availability of elements such as nitrogen or phosphorus on the dynamics of indigenous or introduced *Frankia* populations in plants and soil. Molecular methods may be used to study the ecology of indigenous populations of *Frankia* but also the competitive abilities of introduced strains in order to improve the symbiosis for economic purposes. These studies may lead to advancements in the management of actinorhizal plants and *Frankia*, provided specific *Frankia* populations can be attributed with silviculturally beneficial features. Such features include persistence and growth in soil, competition with less efficient *Frankia* populations for nodule formation, prompt and efficient nodule formation, and ultimately superior N_2-fixing capacity.

2.6. References

1. **Schwintzer, C.R. and J.D. Tjepkema**. 1990. The Biology of *Frankia* and Actinorhizal Plants. San Diego, CA, Academic Press.

2. **Baker, D.D.** 1987. Relationships among pure-cultured strains of *Frankia* based on host specificity. Physiologia Plantarum 70: 245-248.

3. **Bosco, M., M.P. Fernandez, P. Simonet, R. Materassi and P. Normand**. 1992. Evidence that some *Frankia* spp. strains are able to cross boundaries between *Alnus* and *Elaeagnus* host specificity groups. Applied and Environmental Microbiology 58(5): 1569-1576.

4. **Hahn, D., M.J.C. Starrenburg and A.D.L. Akkermans**. 1988. Variable compatibility of cloned *Alnus glutinosa* ecotypes against ineffective *Frankia* strains. Plant and Soil 107: 233-243.

5. **Benson, D.R. and W.B. Silvester**. 1993. Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. Microbiological Reviews 57(2): 293-319.

6. **Huss-Danell, K.** 1997. Actinorhizal symbioses and their N_2 fixation. New Phytologist 136: 375-405.

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

8. **Huss-Danell, K. and D.D. Myrold**. 1994. Intrageneric variation in nodulation of *Alnus*: consequences for quantifying *Frankia* nodulation units in soil. Soil Biology and Biochemistry 26: 525-531.

9. **Myrold, D.D., A.B. Hilger, K. Huss-Danell and K.J. Martin**. 1994. Use of molecular methods to enumerate *Frankia* in soil. In: Beyond the biomass, Eds.: K. Ritz, J. Dighton, and K.E. Giller, John Wiley & Sons, Chichester, UK: 127-136.

10. **Smolander, A.** 1990. *Frankia* populations in soils under different tree species - with special emphasis on soil under *Betula pendula*. Plant and Soil 121: 1-10.

11. **Maunuksela, L., K. Zepp, T. Koivula, J. Zeyer, K. Haahtela and D. Hahn.** 1999. Analysis of *Frankia* in three soils devoid of actinorhizal plants. FEMS Microbiology Ecology 28: 11-21.

12. **Simonet, P., M.C. Grossjean, A.K. Misra, S. Nazaret, B. Cournoyer and P. Normand.** 1991. *Frankia* genus-specific characterization by polymerase chain reaction. Applied and Environmental Microbiology 57(11): 3278-3286.

13. **Normand, P. and C. Chapelon.** 1997. Direct characterization of *Frankia* and of close phyletic neighbors from an *Alnus viridis* rhizosphere. Physiologia Plantarum 99: 722-731.

14. **Picard, C., C. Ponsonnet, E. Paget, X. Nesme and P. Simonet.** 1992. Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. Applied and Environmental Microbiology 58(9): 2717-2722.

15. **Mirza, M.S., D. Hahn and A.D.L. Akkermans.** 1992. Isolation and characterization of *Frankia* strains from *Coriaria nepalensis*. Systematic and Applied Microbiology 15: 289-295.

16. **Nazaret, S., B. Cournoyer, P. Normand and P. Simonet.** 1991. Phylogenetic relationships among *Frankia* genomic species determined by use of amplified 16S rDNA sequences. Journal of Bacteriology 173(13): 4072-4078.

17. **Simonet, P., M. Bosco, C. Chapelon, A. Moiroud and P. Normand.** 1994. Molecular characterization of *Frankia* microsymbionts from spore-positive and spore-negative nodules in a natural alder stand. Applied and Environmental Microbiology 60(4): 1335-1341.

18. **Nick, G., E. Paget, P. Simonet, A. Moiroud and P. Normand.** 1992. The nodular endophytes of *Coriaria* ssp. form a distinct lineage within the genus *Frankia*. Molecular Ecology 1: 175-181.

19. **Hönerlage, W., D. Hahn, K. Zepp, J. Zeyer and P. Normand.** 1994. A hypervariable 23S rRNA region provides a discriminating target for

specific characterization of uncultured and cultured *Frankia*. Systematic and Applied Microbiology 17: 433-443.

20. **Zepp, K., D. Hahn and J. Zeyer**. 1997. *In-situ* analysis of introduced and indigenous *Frankia* populations in soil and root nodules obtained on *Alnus glutinosa*. Soil Biology and Biochemistry 29(9/10): 1595-1600.

21. **Maggia, L., S. Nazaret and P. Simonet**. 1992. Molecular characterization of *Frankia* isolates from *Casuarina equisetifolia* root nodules harvested in West Africa (Senegal and Gambia). Acta Oecologica 13: 453-461.

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

23. **Nalin, R., A.M. Domenach and P. Normand**. 1995. Molecular structure of the *Frankia* ssp. *nifD-K* intergenic spacer and design of *Frankia* genus compatible primer. Molecular Ecology 4: 483-491.

24. **Cournoyer, B. and P. Normand**. 1994. Characterization of a spontaneous thiostrepton-resistant *Frankia alni* infective isolate using PCR-RFLP of *nif* and *gln*II genes. Soil Biology and Biochemistry 5: 553-559.

25. **Murry, M.A., A.S. Konopka, S.D. Pratt and T.L. Vandergon**. 1997. The use of PCR-based typing methods to assess the diversity of *Frankia* nodule endophytes of the actinorhizal shrub *Ceanothus*. Physiologia Plantarum 99: 714-721.

26. **Murry, M.A., D. Zhang, M. Schneider and F.J. de Bruijn**. 1995. Use of repetitive sequences and the polymerase chain reaction (rep-PCR) to fingerprint the genomes of *Frankia* isolates. Symbiosis 19: 223-240.

27. **Sellstedt, A., B. Wullings, U. Nyström and P. Gustafsson**. 1992. Identification of *Casuarina-Frankia* strains by use of polymerase chain reaction (PCR) with arbitrary primers. FEMS Microbiology Letters 93: 1-6.

28. **Hahn, D., R. Kester, M.J.C. Starrenburg and A.D.L. Akkermans**. 1990. Extraction of ribosomal RNA from soil for detection of *Frankia* with oligonucleotide probes. Archives of Microbiology 154: 329-335.

29. **Hahn, D., M. Dorsch, E. Stackebrandt and A.D.L. Akkermans**. 1989. Synthetic oligonucleotide probes for identification of *Frankia* strains. Plant and Soil 118: 211-219.

30. **Mirza, M.S., S. Hameed and A.D.L. Akkermans**. 1994. Genetic diversity of *Datisca cannabina*-compatible *Frankia* strains as determined by sequence analysis of the PCR-amplified 16S rRNA gene. Applied and Environmental Microbiology 60(7): 2371-2376.

31. **Zepp, K., D. Hahn and J. Zeyer**. 1997. Evaluation of a 23S rRNA insertion as target for the analysis of uncultured *Frankia* populations in root nodules of alders by whole cell hybridization. Systematic and Applied Microbiology 20: 124-132.

32. **Simonet, P., P. Normand, M.A. and R. Bardin**. 1990. Identification of *Frankia* strains in nodules by hybridization of polymerase chain reaction products with strain specific oligonucleotide probes. Archives of Microbiology 153: 235-240.

33. **Prin, Y., F. Mallein-Gerin and P. Simonet**. 1993. Identification and localization of *Frankia* strains in *Alnus* nodules by *in situ* hybridization of *nif*H mRNA with strain-specific oligonucleotide probes. Journal of Experimental Botany 44(261): 815-820.

34. **Simonet, P., N.T. Le, E.T. Cross and R. Bardin**. 1988. Identification of *Frankia* strains by direct DNA hybridization of crushed nodules. Applied and Environmental Microbiology 54(10): p2500-2503.

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

36. **Mirza, M.S., D. Hahn, S.V. Dobritsa and A.D.L. Akkermans**. 1994. Phylogenetic studies on uncultured *Frankia* populations in nodules of *Datisca cannabina*. Canadian Journal of Botany 40: 313-318.

37. **Benson, D.R., D.W. Stephens, M.L. Clawson and W.B. Silvester**. 1996. Amplification of 16S rRNA genes from *Frankia* strains in root nodules of *Ceanothus griseus*, *Coriaria arborea*, *Discaria toumatoul*, and *Purshia tridentata*. Applied and Environmental Microbiology 62(8): 2904-2909.

38. **Clawson, M.L., M. Caru and D.R. Benson**. 1998. Diversity of *Frankia* spp. strains in root nodules of plants from the Elaeagnaceae and Rhamnaceae. Applied and Environmental Microbiology 64: 3539-3543.

39. **Navarro, E., R. Nalin, D. Gauthier and P. Normand**. 1997. The nodular microsymbionts of *Gymnostoma* ssp. are *Elaeagnus*-infective *Frankia* strains. Applied and Environmental Microbiology 63(4): 1610-1616.

40. **Ramirez-Saad, H., J. Janse and A.D.L. Akkermans**. 1998. Root nodules of *Ceanothus caerulens* contain both the N_2-fixing *Frankia* endophyte and a phylogenetically related Nod-/Fix- actinomycete. Canadian Journal of Microbiology 44: 140-148.

41. **Wolters, D.J., C.v. Dijk, E.G. Zoetendal and A.D.L. Akkermans**. 1997. Phylogenetic characterization of ineffective *Frankia* in *Alnus glutinosa* (L.) Gaertn. nodules from wetland soil inoculants. Molecular Ecology 6: 971-981.

42. **Hahn, D., M.J.C. Starrenburg and A.D.L. Akkermans**. 1990. Oligonucleotide probes that hybridize with rRNA as a tool to study *Frankia* strains in root nodules. Applied and Environmental Microbiology 56(5): 1342-1346.

43. **Baker, D.D. and B.C. Mullin**. 1994. Diversity of *Frankia* nodule endophytes of the actinorhizal shrub *Ceanothus* as assessed by RFLP patterns from single nodule lobes. Soil Biology and Biochemistry 26(5): 547-552.

44. **McEwan, N.R., C.T. Wheeler and J.J. Milner.** 1994. Strain discrimination of cultured and symbiotic *Frankia* by RFLP-PCR. Soil Biology and Biochemistry 5: 541-545.

45. **Nalin, R., P. Normand and A.M. Domenach.** 1997. Distribution and N_2-fixing activity of *Frankia*-strains in relation to soil depth. Physiologia Plantarum 99: 732-738.

46. **Lumini, E., M. Bosco and M.P. Fernandez.** 1996. PCR-RFLP and total DNA homology revealed three related genomic species among broad-host-range *Frankia* strains. FEMS Microbiology Ecology 21: 303-311.

47. **Rouvier, C., Y. Prin, P. Reddell, P. Normand and P. Simonet.** 1996. Genetic diversity among *Frankia* strains nodulating members of the family Casuarinaceae in Australia revealed by PCR and restriction fragment length polymorphism analysis with crushed root nodules. Applied and Environmental Microbiology 62(3): 979-985.

48. **Myrold, D.D. and K. Huss-Danell.** 1994. Population dynamics of *Alnus* infective *Frankia* in a forest soil with and without host trees. Soil Biology and Biochemistry 26: 533-540.

49. **Niner, B.M., J.P. Brandt, M. Villegas, C.R. Marshall, A.M. Hirsch and M. Valdés.** 1996. Analysis of partial sequences of genes coding for 16S rRNA of actinomycetes isolated from *Casuarina equisetifolia* nodules in Mexico. Applied and Environmental Microbiology 62(8): 3034-3036.

50. **Eppard, M., W.E. Krumbein, C. Koch, E. Rhiel, J.T. Staley and E. Stackebrandt.** 1996. Morphological, physiological, and molecular characterization of actinomycetes isolated from dry soil, rocks, and monument surfaces. Archives of Microbiology 166: 12-22.

Seite Leer /
Blank leaf

Chapter 3

In situ analysis of introduced *Frankia* populations in root nodules of *Alnus glutinosa* grown under different water availability

A. Nickel, D. Hahn, K. Zepp, J. Zeyer

Canadian Journal of Botany 77 (1999), 1231-1239

Chapter 3

In situ analysis of introduced *Frankia* populations in root nodules of *Alnus glutinosa* grown under different water availability

3.1. Abstract

The competitive ability for nodulation of *Alnus glutinosa* plants by *Frankia* strains inoculated into soil with indigenous *Frankia* populations was studied at two matric potentials representing "dry" (-0.016 MPa) and "wet" (-0.001 MPa) conditions. In pots kept at a matric potential of -0.001 MPa, nitrate concentrations decreased within 3 weeks more than 10-fold to an average of approx. 200 µmol [g soil dry wt.]$^{-1}$. After 4 months, nitrate concentrations in these pots were 16 ± 16 and 277 ± 328 µmol [g soil dry wt.]$^{-1}$ for non-inoculated and inoculated soils, respectively. At a matric potential of -0.016 MPa, nitrate concentrations for non-inoculated and inoculated soils were 687 ± 491 and 1796 ± 1746 µmol [g soil dry wt.]$^{-1}$, respectively. Inoculated plants always grew better than their non-inoculated counterparts. The largest plants were found on inoculated soil at a matric potential of -0.001 MPa, whereas the smallest plants were found on non-inoculated soil at the same matric potential. At a matric potential of -0.016 MPa, plants grown on non-inoculated soil were not as high as those grown on inoculated soil and slightly chlorotic indicating that the high level of nitrate in the soil was not providing optimal plant growth conditions. The number of nodule lobes formed on plants was not significantly different in all treatments, though size and weight of lobes differed. Nodules from plants grown on inoculated soils always harbored vesicle-producing *Frankia* populations while in nodules from plants grown on non-inoculated soils only frankiae with distorted vesicles or no frankiae at all were detected. All strains in nodules from plants grown on non-inoculated soil were of *Alnus* host infection group IIIa. Nodules from plants grown on soil inoculated with strains Arl3 (group IIIa), Ag45/Mut15 (group IV), and AgB1.9 (group I) were also infected by *Frankia* strain Ag45/Mut15. These results indicate that by inoculation

Frankia populations could be established in root nodules under conditions that did not favor vesicle formation in nodules formed by the indigenous *Frankia* population. Inoculation even in soils with high nitrogen content might therefore be an appropriate strategy to enhance plant growth.

3.2. Introduction

Actinorhizal plants are characterized by their ability to form root nodules in symbiosis with the N_2-fixing actinomycete *Frankia* which enables them to grow on sites with restricted nitrogen availability [1]. Economically, actinorhizal plants are interesting for reforestation and reclamation of depauperate, nitrogen-limiting soils, especially in developing countries in subtropical regions in which, in addition to their use for reforestation and reclamation of poor soils, *Casuarina* species are an important source of firewood [2], [3]. In temperate regions *Alnus* species have the highest potential for use in forestry [4, 5]. They are used as nurse trees in mixed plantations with valuable tree species, i.e. by interplanting them with suitable tree crops such as walnut, for production of fuelwood and as source of timber in monocultures [5-8].

The efficiency of root nodule formation on actinorhizal plants is largely determined by environmental factors such as the soil pH [9-11], the soil matric potential [12, 13], and the availability of elements such as nitrogen [14, 15] or phosphorus [16, 17], but also by the source of genotypes of both partners of this symbiosis [18, 19]. 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* [18, 20, 21]. Criteria such as nitrogen-fixing capacity and compatibility of *Frankia* strains and the ability of introduced strains to form nodules promptly, to persist in soil and to compete with indigenous *Frankia* populations must be considered for efficient inoculation programs with *Frankia* strains on alders.

The aim of our study was to investigate the competitive ability of introduced *Frankia* strains with indigenous *Frankia* populations in soil for nodulation on *A. glutinosa* plants. Non-inoculated soil or soil inoculated with *Frankia* strains ArI3 (group IIIa), Ag45/Mut15 (group IV), and AgB1.9 (group I) were planted with *A.*

glutinosa seedlings and kept at two matric potentials representing "dry" (-0.016 MPa) and "wet" (-0.001 MPa) conditions found at natural stands of *A. glutinosa*. Matric potentials were maintained for 4 months and their impact determined with respect to soil-, plant- and nodulation-parameters. In root nodules obtained on plants grown on inoculated and non-inoculated soils, *Frankia* populations were analyzed by *in situ* hybridization [22, 23].

3.3. Material and Methods

3.3.1. Experimental set up

Surface samples (down to a depth of 20 cm) were collected from a sandy loam at a natural stand of *A. glutinosa* (Ettiswil, Switzerland) [22]. This soil was characterized by high nitrate concentrations (6 to 7 mM), a low content of organic material (0.02%), a high matric potential (-0.016 MPa) and the presence of *Frankia* subgroups IIIa, IIIb, and IV of the *Alnus* host infection group. At the natural site, however, nodules were only formed by subgroup IIIa [22, 23]. Freshly sampled soil was cleared of larger particles, e.g. roots and stones, sieved (mesh size 5 mm), and stored at 12°C. After 4 weeks of storage, a part of the soil was inoculated with a mixture of pure cultures of *Frankia* strains AgB1.9, Arl3, and Ag45/Mut15. *Frankia* strains were grown for 4 weeks in P+N-medium [24] containing sodium propionate and ammonium chloride as C- and N-source, respectively. Cultures were harvested by centrifugation, washed twice in phosphate buffered saline (PBS, composed of 0.13 M NaCl, 7 mM Na_2HPO_4 and 3 mM NaH_2PO_4, pH 7.2 in water) [25] and homogenized in PBS by repeated passages through a needle (0.6 mm in diameter) with a sterile syringe [26]. Cell numbers were calculated from fresh weight determination. Five ml of the homogenized cultures were sprayed onto thin layers of 800-g-subsamples of soil [fresh wt.] followed by careful mixing to achieve an even distribution of *Frankia* strains each at an estimated density of 10^7 cells [g soil dry wt.]$^{-1}$. Soil samples kept non-inoculated were only mixed.

Each 800 g of inoculated (n=40) and non-inoculated (n=20) soil samples were filled into 800 cm^3 pots. Pots were planted with approximately 4-week-old seedlings of *A. glutinosa* (L.) Gaertn. that had been germinated and grown in

Perlite supplemented with a modified Heller salt solution [27] containing 0.075 µM nitrate as nitrogen-source at pH 5.4 [28]. Plants were maintained in a growth chamber with a thermoperiod of 24/18°C and a photoperiod of 16/8 h day/night. Half of the samples, i.e. 20 pots with inoculated and 10 pots with non-inoculated soil were adjusted to and maintained at a matric potential of -0.016 MPa, the other half at -0.001 MPa. The matric potential was separately maintained in every pot via suction cups and controlled at the end of the experiment by determination of water contents and comparison to a moisture release characteristic determined for the original soil at the appropriate bulk density. Plants in pots were grown under natural light and temperature conditions in the greenhouse for 4 months (March 14 to July 14).

3.3.2. Analysis of soil parameters

Concentrations of NO_3^-, NO_2^-, SO_4^{2-}, PO_4^{3-}, and Cl^- were analyzed in pore water of soil samples obtained at the beginning of the study, after 1, 2, and 3 weeks of growth, and at the end of the experiment after 4 months. Pore water was obtained by centrifugation of approx. 12 g of soil filled into 5 ml plastic syringes which were plugged with silane treated glass wool (Supelco Inc., Bellefonte, USA). The filled syringes were placed in 15 ml Falcon tubes and centrifuged at 4°C and 2500 x g for 10 minutes. 15-µl-samples of pore water were analyzed by ion chromatography (Dionex DX-100 ion chromatograph equipped with an IonPac AS4A-SC column; Dionex, Sunnyvale, USA) using an eluent of 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ [29]. Data from ion chromatography were analyzed with Chrom-Card for Windows (Fison Instruments, Rodano, Italy) [29]. Concentrations in pore water (µM) were correlated to water contents and expressed in µmol [g soil dry wt.]$^{-1}$.

3.3.3. Analysis of plant parameters

Plant height was monitored monthly. At the end of the experiment after 4 months, shoots, roots and nodules of the plants were harvested separately. Shoots and roots were dried at 105°C for 24 h to determine dry weights and to calculate shoot/root ratios. C/N ratios in leaves were determined after the analysis of C and

N contents in dried and ground leaves using a CHNS-932 analyzer (Leco, Kirchheim, Germany). Numbers of nodule lobes were counted and fresh weights of nodules determined.

3.3.4. Analysis of *Frankia* populations in root nodules

For the analysis of *Frankia* populations in root nodules, all nodules were harvested, split into lobes and fixed in 4% paraformaldehyde in PBS at 4°C for 16 h [30]. Lobes were subsequently washed in PBS and about two-third of the lobes ground in a mortar. Lobe homogenates and remaining nodule lobes were stored in 96% ethanol at -20°C [30]. All remaining nodule lobes were covered with and soaked in embedding medium (No. 350100; Microm, Walldorf, Switzerland) for 2 days and subsequently sectioned longitudinally through the main axis in a HM 500 OM cryostat (Microm). Sections between 10 and 14 µm as well as 3-µl-samples of lobe homogenates were air-dried on gelatin-coated slides (0.1% gelatin, 0.01% $KCr(SO_4)_2$) for at least 2 h [31]. After dehydration in 50, 80 and 96% ethanol for 3 min each, the preparations were pretreated with SDS/DTT (10 mg ml^{-1} SDS, 50 mM Dithiothreitol (DTT, Fluka) in water, freshly prepared) at 65°C for 30 min followed by an incubation with lysozyme (Fluka, Buchs, Switzerland, 1 mg corresponding to 37 320 U dissolved in 1 ml of 100 mM Tris/HCl, pH 7.5, 5 mM EDTA) at 37°C for 10 min [22]. Afterwards, the samples were rinsed with distilled water and dehydrated as described above.

Oligonucleotide probes targeting 16S rRNA of the domain Bacteria (Eub338; [32] or specific sequences on the 23S rRNA insertion of *Frankia* strains AgB1.9 (probe B1.9; $5'$ACC ACC TCA ACC CCC GAA), Arl3 (probe 23Arl3) [23], and Ag45/Mut15 (probe 23Mut(II)) [23] representing *Alnus* host infection groups I, IIIa, and IV, respectively [33], were synthesized with a primary amino group at the 5' end (C6-TFA, MWG, Ebersberg, Germany). The fluorescent dye Cy3 (Amersham, Zurich, Switzerland) was covalently bound to the amino group of the oligonucleotide probe. The dye-oligonucleotide conjugate (1:1) was purified from unreacted components and stored at -20°C in distilled water at a concentration of 25 ng μl^{-1} [32]. This solution was amended with the DNA specific dye 4'6-

diamidino-2-phenylindole (DAPI) (Sigma, Buchs, Switzerland) to give a final concentration of 10 µg ml^{-1}.

Hybridizations were performed in 9 µl of hybridization buffer (900 mM NaCl, 20 mM Tris/HCl, 0.01% SDS, pH 7.2) in the presence of 30% formamide [34] and 1 µl of oligonucleotide probe (25 ng) at 42°C for 2 h [22, 23]. After hybridization, the slides were washed in hybridization buffer without formamide at 48°C for 20 min, rinsed with distilled water, and air-dried. Preparations were mounted with Citifluor solution (Citifluor, Canterbury, UK) and examined with a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) fitted for epifluorescence detection with a high-pressure mercury bulb (50 W). DAPI-stained bacteria were analyzed with filter set 02 (Zeiss; G365, FT395, LP420), whereas binding of Cy3-labeled probes was detected with filter set HQ-Cy3 (AHF Analysentechnik, Tübingen, Germany; G535/50, FT565, BP610/75).

3.3.5. Statistical analyses

All data were expressed as mean ± standard deviation and assessed by multiple pairwise comparisons with Tukeys HSD test (SYSTAT). The significance level was set at $p < 0.05$.

3.4. Results

3.4.1. Soil parameters

Differences in water contents developed between non-inoculated and inoculated pots kept at the same matric potential. The water contents of non-inoculated soils with matric potentials of -0.001 and -0.016 MPa were, respectively, 14% and 43% higher than inoculated soils of the same matric potentials (Table 3.1). At the end of the experiment after 4 months, pots kept at a matric potential of -0.001 MPa had an average water content of 29 ± 3%, those kept at -0.016 MPa an average water content of 16 ± 4% which corresponded to matric potentials of -0.005 MPa and < -0.02 MPa, respectively. At the beginning of the experiment the pore water had a pH of 7.1. During and at the end of the experiment, no major changes in pH were observed (Table 3.1). Nitrate concentrations in the pore water changed

significantly during the experiment. In pots where soil matric potential was maintained at -0.001 MPa, nitrate concentration in pore water decreased 13-fold within 3 weeks to an average of 200 µmol [g soil dry wt.]$^{-1}$. After 4 months, the nitrate concentrations in these pots, when non-inoculated or inoculated with *Frankia*, were 1% and 11% of the original value, respectively (Table 3.1). This compares with significantly higher nitrate concentrations of 26% and 69% of the original value that remained in non-inoculated and inoculated pots, respectively, when soil matric potential was -0.016 MPa. Concentrations of nitrite increased from below the detection limit (1 µM) at the beginning of the experiment to values of up to 35 µmol [g soil dry wt.]$^{-1}$. Sulfate concentrations (191 µmol [g soil dry wt.]$^{-1}$) remained in the same range in pots with a matric potential of -0.016 MPa, but were reduced about 3-fold in pots kept at a matric potential of -0.001 MPa (Table 3.1). In all pots chloride concentrations remained in the same range of 311 ± 414 µmol [g soil dry wt.]$^{-1}$. Phosphate concentrations were always below the detection limit at 20 µM.

Table 3.1. Soil parameters at the beginning and at the end of the growth experiment (non-inoculated and inoculated soils)

Soil parameters[d/e]		Matric potential (-0.001 MPa)		Matric potential (-0.016 MPa)	
	Initial conditions[a]	Non-inoculated[b]	Inoculated[c]	Non-inoculated[b]	Inoculated[c]
Water content (%)	23 ± 1	32 ± 1	28 ± 2	20 ± 1	14 ± 3
pH (in water)	7.1 ± 0.1	7.4 ± 0.2	7.3 ± 0.2	7.2 ± 0.2	7.3 ± 0.2
NO_3^- (µmol [g soil dry wt.]$^{-1}$)	2609 ± 1906	16 ± 16	277 ± 329	687 ± 491	1796 ± 1746
NO_2^- (µmol [g soil dry wt.]$^{-1}$)	< 1	1 ± 3	11 ± 27	8 ± 23	35 ± 64
SO_4^{2-} (µmol [g soil dry wt.]$^{-1}$)	191 ±101	58 ± 97	83 ± 54	214 ± 220	138 ± 94
Cl^- (µmol [g soil dry wt.]$^{-1}$)	311 ± 414	136 ± 88	244 ± 208	407 ± 406	313 ± 226

[a] soil "Ettiswil" at the beginning of the experiment (n=5)

[b] soil "Ettiswil" after 4 months (n=10)

[c] soil "Ettiswil" after 4 months, inoculated with *Frankia* strains ArI3, Ag45/Mut15 and AgB1.9 at a density of each approx. 10^7 cells [g soil fresh wt.]$^{-1}$ (n=20)

[d] concentrations of phosphate were always below the detection limit of 20 µM

[e] only values for water content and nitrate concentrations are significantly different ($p = 0.05$) between non-inoculated and inoculated soils at the same matric potential

3.4.2. Plant parameters

Monthly plant height measurements showed a faster growth of inoculated plants compared to non-inoculated plants (Fig. 3.1). After 4 months of growth, plants on the non-inoculated soil kept at a matric potential of -0.001 MPa were the smallest plants with 18 ± 10 cm (Table 3.2). Those on inoculated soil at the same matric potential were 3-fold larger, and those on non-inoculated and inoculated soil at a matric potential of -0.016 MPa about 1.5-fold and 2-fold larger, respectively. The statistically significant differences of the latter, however, were just coming up towards the end of the experiment (Fig. 3.1). Comparable patterns to plant height measurements were found for plant dry weights (Table 3.2). The highest values for shoot and root dry weights were obtained with plants growing on inoculated soil at a matric potential of -0.001 MPa, which were about 4-fold higher than that for plants on non-inoculated soil at the same matric potential (1.5 ± 0.9 g [dry wt.]). Plants grown at a matric potential of -0.016 MPa showed values of 2.5 ± 0.8 and 3.8 ± 1.8 g [dry wt.], again with higher values for the inoculated soil.

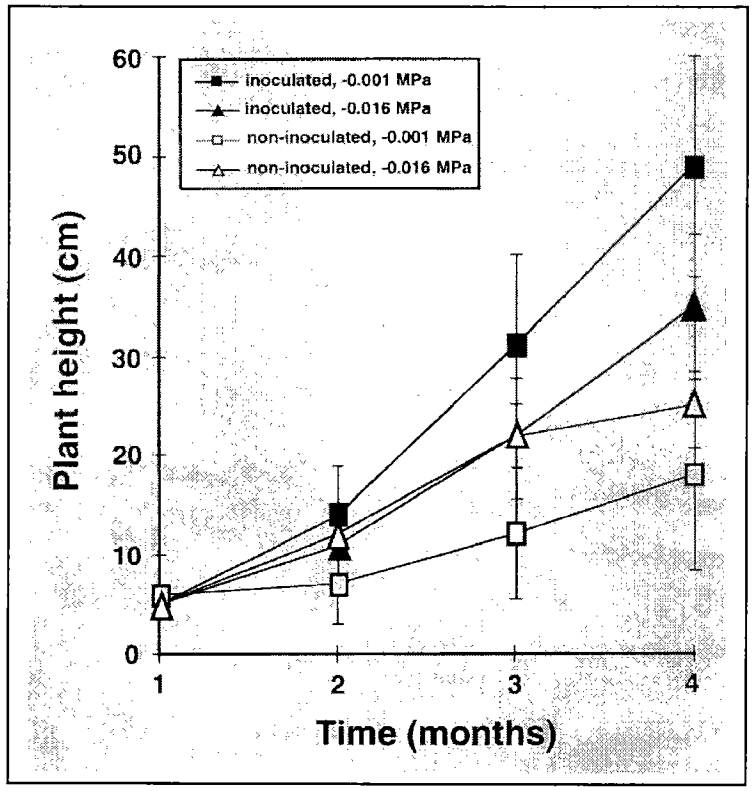


Figure 3.1. Height of *A. glutinosa* seedlings grown on inoculated or non-inoculated soils at two matric potentials (-0.001 and -0.016 MPa), respectively.

Root/shoot ratios between plants grown at matric potentials of -0.001 and -0.016 MPa, respectively, were not significantly different. No differences in root/shoot ratios were obtained between plants grown on inoculated and non-inoculated soils (Table 3.2). The content of carbon in leaves was similar in all treatments (47 ± 1%), while the content of nitrogen differed. Highest nitrogen contents were obtained in leaves of plants grown on inoculated soil (2.9 ± 0.4 and 3.0 ± 0.3% at matric potentials of -0.001 and -0.016 MPa, respectively). Leaves from plants grown on non-inoculated soils were chlorotic and consequently had much lower nitrogen contents (2.2 ± 0.4 and 1.9 ± 0.2% at matric potentials of -0.001 and -0.016 MPa, respectively). Consequently, C/N ratios for leaves of plants grown on inoculated soils were lower than for leaves of plants grown on non-inoculated soils.

Table 3.2. Plant parameters after 4 months of growth

Plant parameters [c]	Matric potential (-0.001 MPa)		Matric potential (-0.016 MPa)	
	Non-inoculated[a]	Inoculated[b]	Non-inoculated[a]	Inoculated[b]
Plant height (cm)	18 ± 10	49 ± 11	28 ± 4	35 ± 8
Plant weight (g [dry wt.])	1.5 ± 0.9	5.9 ± 3.2	2.5 ± 0.8	3.7 ± 1.8
Shoot (g [dry wt.])	0.8 ± 0.5	3.2 ± 1.8	1.5 ± 0.3	2.1 ± 1.0
Root (g [dry wt.])	0.7 ± 0.5	2.7 ± 1.5	1.0 ± 0.5	1.6 ± 0.9
Root/shoot ratio	0.9 ± 0.4	0.9 ± 0.4	0.7 ± 0.3	0.7 ± 0.3
N-content (%)	2.2 ± 0.4	2.9 ± 0.4	1.9 ± 0.2	3.0 ± 0.3
C/N ratio of leaves	21 ± 4	16 ± 2	25 ± 3	16 ± 2

[a] soil "Ettiswil" (n=10)

[b] soil "Ettiswil", inoculated with *Frankia* strains Arl3, Ag45/Mut15 and AgB1.9 at a density of each approx. 10^7 cells [g soil fresh wt.]$^{-1}$ (n=20)

[c] only values for plant height, plant weight, shoot weight and C/N ratio are significantly different ($p = 0.05$) between non-inoculated and inoculated soils at the same matric potential

3.4.3. Populations of *Frankia* in root nodules

With an average of about 20 lobes per plant, the number of nodule lobes formed on plants was not significantly different in all treatments, though size and weight of lobes differed (Table 3.3). Lobes formed on plants grown on inoculated soil at a matric potential of -0.001 MPa had a total weight of 358 ± 211 mg [fresh wt.] and an average weight per lobe of 22 ± 11 mg [fresh wt.]. These values were reduced 18-fold and 11-fold, respectively, on plants grown on non-inoculated soil of the same matric potential. At a matric potential of -0.016 MPa, lobes on plants grown on inoculated soil had a total weight of 166 ± 116 mg [fresh wt.] and an average lobe weight of 9 ± 4. Both values were reduced about 4-fold for lobes on plants grown on non-inoculated soil (Table 3.3).

Nodule lobes from plants grown on inoculated soils usually contained *Frankia* populations though occasionally nodule lobes without *Frankia* were found. All lobes from plants grown on inoculated soils contained N_2-fixing frankiae, except for one in which the non-N_2-fixing *Frankia* strain AgB1.9 was detected (Table 3.3). In these treatments *Frankia* strains Ag45/Mut15 and Arl3 were identified in high percentages with probes 23Mut(II) and 23Arl3, respectively. Nodule lobes on plants grown at a matric potential of -0.001 MPa contained comparable percentages of Ag45/Mut15 (45 ± 27%) and Arl3 (49 ± 28%), those from plants grown at a matric potential of -0.016 MPa had slightly less nodules infected by Ag45/Mut15 (38 ± 25) than by Arl3 (62 ± 24).

On non-inoculated soils, about 90% of the lobes on plants grown at a matric potential of -0.016 MPa and 40% of those on plants grown at a matric potential of -0.001 MPa did not contain filaments, vesicles or spores typical for frankiae. Vesicles in the remaining nodules were usually much smaller than those in nodules from plants on the inoculated soils (Fig. 3.2). The majority of plants grown at a matric potential of -0.016 MPa (8 out of 10) did not have any frankiae-containing nodules. In contrast, 8 out of 10 plants grown at a matric potential of -0.001 MPa had nodules with frankiae though vesicles were usually distorted. *Frankia* populations in vesicle-containing lobes of plants grown on non-inoculated soils at either matric potential were all detectable with probe 23Arl3 (55 ± 37% and 13 ± 28%). None of the other *Frankia*-specific probes (23B1.9, and 23Mut(II)) showed hybridization signals.

Table 3.3. Nodulation parameters

Nodulation parameters [f]	Matric potential (-0.001 MPa)		Matric potential (-0.016 MPa)	
	Non-inoculated[a]	Inoculated[b]	Non-inoculated[a]	Inoculated[b]
Number of lobes	19 ± 20	18 ± 12	30 ± 28	20 ± 11
Total lobe weight (mg [fresh wt.])	20 ± 35	358 ± 211	38 ± 43	166 ± 116
Average lobe weight (mg [fresh wt.])	2 ± 4	22 ± 11	2 ± 1	9 ± 4
Group IIIa (%)	55 ± 37	49 ± 28	13 ± 28	62 ± 24
Group IV (%)	0	45 ± 27	0	38 ± 25
Group I (%)	0	< 1[c]	0	0
Lobes without *Frankia* (%)	43 ± 36	2 ± 5[d]	87 ± 28	1 ± 2[e]

[a] soil "Ettiswil" (n=10)

[b] soil "Ettiswil", inoculated with *Frankia* strains ArI3, Ag45/Mut15 and AgB1.9 at a density of each approx. 10^7 cells [g soil fresh wt.]$^{-1}$ (n=20)

[c] detection in only 1 nodule

[d] six nodules without *Frankia* on five plants

[e] two nodules without *Frankia* on one plant

[f] all values except for the number of lobes are significantly different ($p = 0.05$) between non-inoculated and inoculated soils at the same matric potential

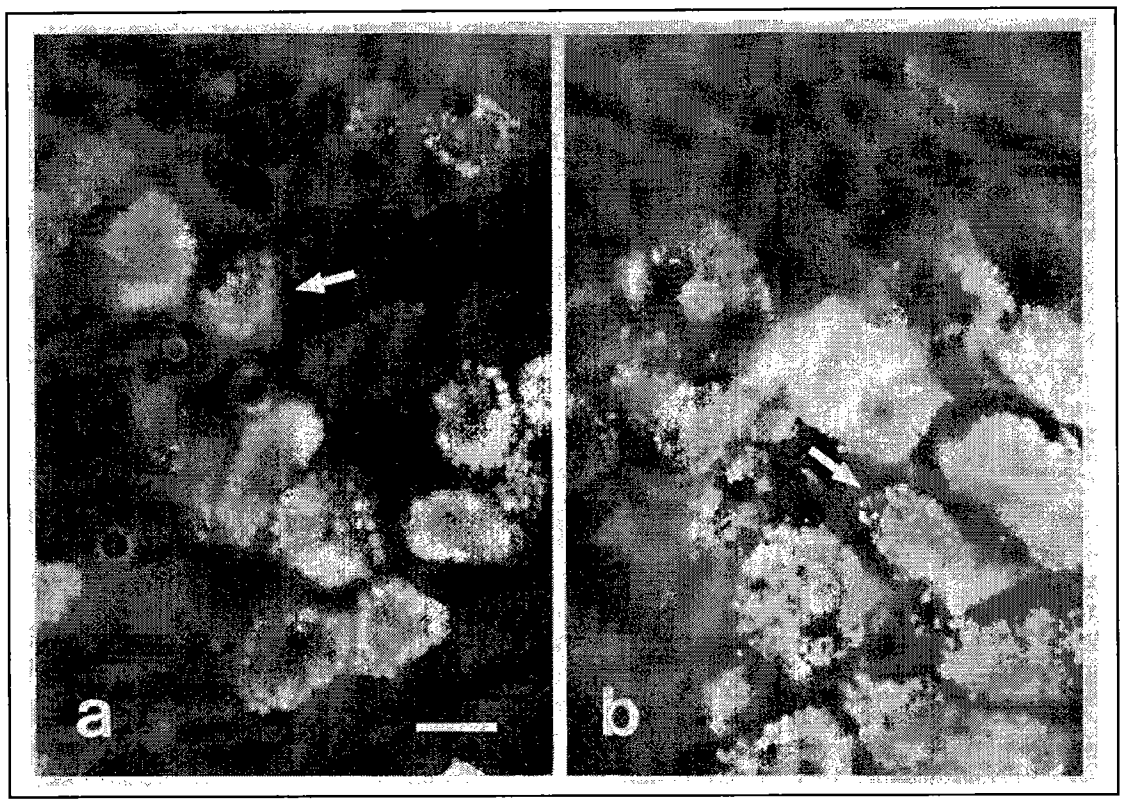


Figure 3.2. Section through nodules of *A. glutinosa* either grown on inoculated soil showing normal vesicles (a) or on non-inoculated soil showing distorted vesicles (b). *Frankia* cells were detected by *in situ* hybridization with fluorescent (Cy3-labeled) oligonucleotide probe 23Arl3. Bar represents 25 µm.

3.5. Discussion

The major set up of the experiment was based on the maintenance of different matric potentials for two sets of plants growing on soils being non-inoculated or inoculated with *Frankia* strains. Although the aim to simulate the matric potentials found at two natural stands of *A. glutinosa* was not achieved, different growth conditions still simulating "dry" and "wet" environmental conditions were obtained. The establishment of "dry" and "wet" environmental conditions was demonstrated by changes in concentration of soil chemicals, particularly nitrate, in soils with different matric potentials. At a matric potential of -0.001 MPa, nitrate concentrations decreased by one order of magnitude within the first three weeks of incubation resulting in nitrogen-limited growth conditions for plants already at this time. The loss of nitrate could be due to high denitrification activity resulting

from the high water content and the concomitant reduction of O_2 availability in these soils. The establishment of at least partially anaerobic conditions is supported by the decrease of sulfate concentrations. At a matric potential of -0.016 MPa, nitrate concentrations decreased in non-inoculated soils to approximately half the original concentration, whereas in soil inoculated with *Frankia* nitrate concentrations remained comparable to those at the beginning of the experiment. At this matric potential, nitrogen availability was initially not limiting plant growth. However, towards the end of the experiment after 4 months leaves on plants grown on non-inoculated soil became slightly chlorotic suggesting a reduced nitrogen supply [12, 26] although nitrate concentrations in soil were still quite high. Plant growth of *A. glutinosa* and nodulation by *Frankia* populations in non-inoculated and inoculated soils kept at two different matric potentials might therefore not only be influenced by the matric potentials but also by different redox conditions, the availability of O_2 and the availability of nitrate as a source of nitrogen.

In our study, inoculated plants always grew better than their non-inoculated counterparts indicating a significant effect of inoculation on plant growth independent of the environmental conditions. Although the number of nodule lobes was not significantly different on plants grown on non-inoculated or inoculated soils, large differences in size and weight of lobes as well as in the inhabiting *Frankia* populations were obtained. Lobes from plants grown on non-inoculated soils were always much smaller than those from plants grown on inoculated soils. The *Frankia* populations in these lobes hybridized only to probe 23Arl3 targeting the *Alnus* host infection group IIIa, confirming earlier studies on field-collected nodules [23] and nodules from a previous competition experiment [22]. In those studies nodules usually contained vesicle-producing frankiae. In contrast, only nodules with distorted vesicles or no frankiae at all were found in our study indicating that, even under conditions of limited nitrogen availability for plants, the indigenous *Frankia* population only formed non-functional nodules at both matric potentials. Under the same environmental conditions, nodules from plants grown on inoculated soils always contained vesicle-producing *Frankia* populations. Due to the lack of nitrogen-deficiency symptoms on the host plants grown at both matric potentials, the vesicles seemed to be functional. Inoculation

with the N_2-fixing *Frankia* strains Arl3 and Ag45/Mut15 demonstrated that both strains outcompeted the indigenous *Frankia* populations showing comparable competitive abilities to each other at both matric potentials.

The result of the inoculation, however, might be influenced by the experimental set up. Comparative analysis of nodulation units with genomic units of soils determined by the PCR-MPN technique using nested PCR [35, 36] indicated that only a small portion of the total population of *Frankia* was able to nodulate. It was suggested that the nodulation capacity of a soil was controlled largely by the physiological status of the *Frankia* populations rather than by the total population size [35]. This suggestion was 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 [22, 23, 37]. The inoculated strains which are physiologically active pure cultures therefore might have a competitive advantage over the indigenous populations which might be largely inactive. Both strains Arl3 and Ag45/Mut15 form nodules promptly, whereas the non-nitrogen-fixing strain AgB1.9, and probably the indigenous *Frankia* population, require much more time for nodule formation [28]. It was shown that a systemic inhibition of nodulation operated in plants of *A. incana* 3 to 6 days after an initial infection with frankiae [38]. A similar effect was observed on plants of *Hippophaë rhamnoides* providing evidence that sea buckthorn had an active, systemic mechanism for feedback control of nodulation that suppressed further nodule formation and prevented excessive nodulation [39].

These results indicate that by inoculation *Frankia* populations could be established in root nodules under conditions that did obviously not favor efficient nitrogenase activity in nodules formed by the indigenous *Frankia* population. Inoculation even in soils with high nitrogen-contents might therefore be an appropriate strategy to enhance plant growth when soils contain only poorly effective frankiae.

3.6. References

1. **Bond, G.** 1983. Taxonomy and distribution of non-legume nitrogen-fixing systems. In: Biological nitrogen fixation in forest ecosystems: foundations and applications, Eds.: G.C. Gordon and C.T. Wheeler, Nijhoff/Junk Publishers, The Hague: 55-87.

2. **Dawson, J.O.** 1986. Actinorhizal plants: Their use in forestry and agriculture. Outlines in Agriculture 15: 202-208.

3. **Wheeler, C.T., J.E. Hooker, A. Crowe and A.M.M. Berry**. 1986. The improvement and utilization in forestry of nitrogen fixation by actinorhizal plants with special reference to *Alnus* in Scotland. Plant and Soil 90: 393-406.

4. **Gordon, J.C. and J.O. Dawson**. 1979. Potential uses of nitrogen-fixing trees and shrubs in commercial forestry. Botanical Gazette 140 (Suppl.): 88-90.

5. **Gordon, J.C.** 1983. Silvicultural systems and biological nitrogen fixation. In: Biological nitrogen fixation in forest ecosystems: foundations and applications, Eds.: J.C. Gordon and C.T. Wheeler, Nijhoff/Junk Publishers, The Hague: 1-6.

6. **Fessenden, R.J.** 1979. Use of actinorhizal plants for land reclamation and amenity planting in the U.S.A. and Canada. In: Symbiotic nitrogen fixation in the management of temperate forests, Eds.: J.C. Gordon, C.T. Wheeler, and D.A. Perry, Corvallis State University, Corvallis, Oregon: 403-419.

7. **Teissier du Cros, E., G. Jung and M. Bariteau**. 1984. Alder-*Frankia* interaction and alder-poplar association for biomass production. Plant and Soil 78: 235-243.

8. **Zavitkovski, J., E.A. Hansen and H.A. McNeel**. 1979. Nitrogen-fixing species in short rotation systems for fiber and energy production. In: Symbiotic nitrogen fixation in the management of temperate forests, Eds.: J.C. Gordon, C.T. Wheeler, and D.A. Perry, Corvallis State University, Corvallis, Oregon: 388-402.

9. **Crannell, W.K., Y. Tanaka and D.D. Myrold.** 1994. Calcium and pH interaction on root nodulation of nursery-grown red alder (*Alnus rubra* Bong.) seedlings by *Frankia*. Soil Biology and Biochemistry 26(5): 607-614.

10. **Griffiths, A.P. and L.H. McCormick.** 1984. Effects of soil acidity on nodulation of *Alnus glutinosa* and viability of *Frankia*. Plant and Soil 79: 429-434.

11. **Zitzer, S.F. and J.O. Dawson.** 1992. Soil properties and actinorhizal vegetation influence nodulation of *Alnus glutinosa* and *Eleagnus angustifolia* by *Frankia*. Plant and Soil 140: 197-204.

12. **Dawson, J.O., D.G. Kowalski and P.J. Dart.** 1989. Variation with soil depth, topographic position and host species in the capacity of soils from an Australian locale to nodulate *Casuarina* and *Allocasuarina* seedlings. Plant and Soil 118: 1-11.

13. **Schwintzer, C.R.** 1985. Effect of spring flooding on endophyte differentiation, nitrogenase activity, root growth and shoot growth in *Myrica gale*. Plant and Soil 87: 109-124.

14. **Kohls, S.J. and D.D. Baker.** 1989. Effects of substrate nitrate concentration on symbiotic nodule formation in actinorhizal plants. Plant and Soil 118: 171-179.

15. **Thomas, K.A. and A.M. Berry.** 1989. Effects of continuous nitrogen application and nitrogen preconditioning on nodulation and growth of *Ceanothus griseus* var *horizontalis*. Plant and Soil 118: 181-187.

16. **Sanginga, N., S.K.A. Danso and G.D. Bowen.** 1989. Nodulation and growth response of *Allocasuarina* and *Casuarina* species to phosphorus fertilization. Plant and Soil 118: 125-132.

17. **Yang, Y.** 1995. The effect of phosphorus on nodule formation and function in the *Casuarina-Frankia* symbiosis. Plant and Soil 176: 161-169.

18. **Hall, R.B., H.S. McNabb, C.A. Maynard and T.L. Green.** 1979. Towards development of optimal *Alnus glutinosa* symbiosis. Botanical Gazette 140 (Suppl.): S120-126.

19. **Prat, D.** 1989. Effects of some pure and mixed *Frankia* strains on seedling growth in different *Alnus* species. Plant and Soil 113: 31-38.

20. **Hilger, A.B., Y. Tanaka and D.D. Myrold**. 1991. Inoculation of fumigated nursery soil increases nodulation and yield of bare-root red alder (*Alnus rubra* Bong.). New Forests 5: 35-42.

21. **Wheeler, C.T., M.K. Hollingsworth, J.E. Hooker, J.D. McNeill, W.L. Mason, A.J. Moffat and L.J. Sheppard**. 1991. The effect of inoculation with either cultured *Frankia* or crushed nodules on nodulation and growth of *Alnus rubra* and *Alnus glutinosa* seedlings in forest nurseries. Forest Ecology and Management 43: 153-166.

22. **Zepp, K., D. Hahn and J. Zeyer**. 1997. *In-situ* analysis of introduced and indigenous *Frankia* populations in soil and root nodules obtained on *Alnus glutinosa*. Soil Biology and Biochemistry 29(9/10): 1595-1600.

23. **Zepp, K., D. Hahn and J. Zeyer**. 1997. Evaluation of a 23S rRNA insertion as target for the analysis of uncultured *Frankia* populations in root nodules of alders by whole cell hybridization. Systematic and Applied Microbiology 20: 124-132.

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

25. **Hahn, D., R.I. Amann, W. Ludwig, A.D.L. Akkermans and K.-H. Schleifer**. 1992. Detection of microorganisms in soil after *in situ* hybridization with rRNA-targeted, fluorescently labeled oligonucleotides. Journal of Genetic and Microbiology 138: 879-887.

26. **Hahn, D., M.J.C. Starrenburg and A.D.L. Akkermans**. 1990. Growth increment of *Alnus glutinosa* upon dual inoculation with effective and ineffective *Frankia* strains. Plant and Soil 122: 121-127.

27. **Heller, R.** 1953. Recherches sur la nutrition minérale des tissues végétaux cultivés in vitro, Paris, France.

28. **Hahn, D., M.J.C. Starrenburg and A.D.L. Akkermans**. 1988. Variable compatibility of cloned *Alnus glutinosa* ecotypes against ineffective *Frankia* strains. Plant and Soil 107: 233-243.

29. **Hess, A., P. Höhener, D. Hunkeler and J. Zeyer**. 1996. Bioremediation of a diesel fuel contaminated aquifer: Simulation studies in laboratory aquifer columns. Journal of Contaminated Hydrologie 23: 329-345.

30. **Hahn, D., R.I. Amann and J. Zeyer**. 1993. Whole-cell hybridization of *Frankia* strains with fluorescence- or digoxigenin-labeled, 16S rRNA-targeted oligonucleotide probes. Applied and Environmental Microbiology 59(6): 1709-1716.

31. **Zarda, B., D. Hahn, A. Chatzinotas, W. Schönhuber, A. Neef, R.I. Amann and J. Zeyer**. 1997. Analysis of bacterial community structure in bulk soil by *in situ* hybridization. Archives of Microbiology 168: 185-192.

32. **Amann, R.I., B.J. Binder, R.J. Olsen, S.W. Chisholm, R. Devereux and D.A. Stahl**. 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Applied and Environmental Microbiology 56: 1919-1925.

33. **Hönerlage, W., D. Hahn, K. Zepp, J. Zeyer and P. Normand**. 1994. A hypervariable 23S rRNA region provides a discriminating target for specific characterization of uncultured and cultured *Frankia*. Systematic and Applied Microbiology 17: 433-443.

34. **Manz, W., R. Amann, W. Ludwig, M. Wagner and K.-H. Schleifer**. 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: Problems and solutions. Systematic and Applied Microbiology 15: 593-600.

35. **Myrold, D.D. and K. Huss-Danell**. 1994. Population dynamics of *Alnus* infective *Frankia* in a forest soil with and without host trees. Soil Biology and Biochemistry 26: 533-540.

36. **Myrold, D.D., A.B. Hilger, K. Huss-Danell and K.J. Martin**. 1994. Use of molecular methods to enumerate *Frankia* in soil. In: Beyond the biomass, Eds.: K. Ritz, J. Dighton, and K.E. Giller, John Wiley & Sons, Chichester, UK: 127-136.

37. **Maunuksela, L., K. Zepp, T. Koivula, J. Zeyer, K. Haahtela and D. Hahn**. 1999. Analysis of *Frankia* in three soils devoid of actinorhizal plants. FEMS Microbiology Ecology 28: 11-21.

38. **Wall, L.G. and K. Huss-Danell**. 1997. Regulation of nodulation in *Alnus incana-Frankia* symbiosis. Physiologia Plantarum 99: 594-600.

39. **Dobritsa, S.V. and S.N. Novik**. 1992. Feedback regulation of nodule formation in *Hippophaë rhamnoides*. Plant and Soil 144: 45-50.

Chapter 4

Use of Molecular and Isotope Techniques to Study the *Frankia* - Alder Symbiosis under Different Environmental Conditions

A. Nickel, O. Pelz, D. Hahn, M. Saurer, R. Siegwolf, J. Zeyer

To be submitted

Seite Leer /
Blank leaf

Chapter 4

Use of Molecular and Isotope Techniques to Study the *Frankia* - Alder Symbiosis under Different Environmental Conditions

4.1. Abstract

An improvement of the efficiency of the symbiosis between *Frankia* and host plant requires basic knowledge on the ecology of *Frankia*. The population structure and the activity of the N_2-fixing actinomycete *Frankia* in symbiosis with *Alnus glutinosa* under different conditions was studied through the combination of stable nitrogen isotope ratio measurements ($\delta^{15}N$) with *in situ* hybridization. Soil containing a natural *Frankia* population consisting of subgroups IIIa, IIIb and IV of the *Alnus* host infection group was divided into four sub-samples: (i) contained only the indigenous *Frankia* population and was defined as control; (ii) was additionally inoculated with *Frankia* pure cultures representing subgroups I, IIIa and IV of the *Alnus* host infection group; (iii) was mixed with dried alder leaves, to study the influence of the dried leaves on *Frankia* growth; (iv) was both inoculated with *Frankia* pure cultures and mixed with dried alder leaves. The soils were incubated in a climate chamber for 6 weeks to study the influence of storage on indigenous and introduced *Frankia* before ^{15}N-nitrate was added and *A. glutinosa* seedlings were planted. After 4 months of growth the highest plants were found on inoculated soil with leaves while the smallest plants grew on control soil. Leaves of control plants were slightly chlorotic, indicating that the high nitrate concentration in the soil did not provide optimal plant growth conditions. Plant height was positively correlated with N_2-fixing activity, the N_2-fixing activity depended on the *Frankia* population in the nodules. The results revealed that: 1. Introduced *Frankia* strains can compete for nodule formation with the indigenous population; 2. Inoculation as well as the addition of dried alder leaves promotes N_2-fixing activity and therefore plant

growth; 3. The addition of dried alder leaves promotes a *Frankia* strain belonging to subgroup IV under the applied conditions.

4.2. Introduction

Actinorhizal plants, e.g. species of *Alnus* (alders), form symbiotic, N_2-fixing root nodules with soil bacteria of the genus *Frankia*. Due to the ability to fix atmospheric nitrogen, the plant is to a large extent independent of soil nitrogen because up to 70%-90% of the total nitrogen assimilated by actinorhizal plants can be provided by the root nodules [1] [2] [3]. In addition, actinorhizal plants can improve soil nitrogen content and availability as well as enhance the growth of neighboring plants by cycling the fixed N into the soil ecosystem [4] [5] [6]. By exploiting these characteristics, actinorhizal plants are widely used in forestry, e.g. for the improvement of disturbed soils, production of timber or fuelwood and for erosion control [2].

Native species of the genus *Alnus* are of special importance in the northern hemisphere due to their wide geographic distribution and their high N_2-fixing potential [7]. There are examples of alder use for timber production [8], production of raw material for paper, chemical industries and energy [9], enhancement of the growth of associated walnut trees [5], reforestation of peat soils [10] and improvement of disturbed soils [4]. Within two years of establishment, N_2-fixing *A. incana* can improve fertility of N-deficient soils by significantly increasing the nitrogen content of the soil [11].

The efficiency of the *Frankia*-alder symbiosis can be improved in two ways: (i) the inoculation with *Frankia* pure cultures [12] [13] [14] and/or (ii) the activation of the indigenous *Frankia* population. It could be shown that the inoculation with selected *Frankia* strains promotes plant growth. Since the nodules are perennial, the positive effect of such inoculations could continue over several years. However, for a long-term effect, the introduced strain should not only remain active in the nodules but also survive in the soil and outcompete the indigenous population while infecting new roots of inoculated plants and seedlings. Therefore, the introduced *Frankia* strains must be able to persist in the soil in a physiologically active status since only the physiologically active

fraction of the *Frankia* soil population is thought to be able to infect roots and form nodules [15]. Another possible way of enhancing the levels of N_2-fixation would be to activate the natural *Frankia* population by adding certain substrates into the soil, which might promote growth and/or N_2-fixing activity of *Frankia*. It is known that *Casuarina* leaves contain substances which promote the growth of *Casuarina*-infective *Frankia* strains [16] and that nodulation can be enhanced by compounds found in seeds of *A. rubra* [17].

Bacterial N_2-fixing in plants is commonly measured through different methods, such as the acetylene reduction assay, xylene-solute assay (or ureide production), total nitrogen difference method, and the use of stable nitrogen isotope ratios (^{15}N /^{14}N). In a comparative study the isotope methods proofed to be the most accurate [18]. The applications of the stable nitrogen isotopes, which use isotopically labeled tracers, are classified into two methods: a direct method with ^{15}N -labeled N_2, and an indirect method, designated as isotope dilution, with ^{15}N -fertilizer [19].

The ^{15}N dilution method is based on the difference between the ^{15}N /^{14}N ratios of nitrogen derived from soil with ^{15}N -fertilizer and atmospheric nitrogen derived from N_2-fixing bacteria. Plants growing in association with N_2-fixing bacteria will accumulate nitrogen with a lower ^{15}N /^{14}N ratio while plants not associated with N_2-fixing bacteria accumulate a higher ^{15}N /^{14}N ratio. This is by virtue of soil N containing a higher ^{15}N /^{14}N ratio than the bacteria-fixed atmospheric N_2 (^{15}N /^{14}N ratio = 0.3663%). Through the uptake of N from bacterially fixed N_2 the ^{15}N /^{14}N ratio of the soil N in plant material is diluted. The non N_2-fixing plants, which accumulate N only from the soil, are used as reference plants to determine the ^{15}N /^{14}N ratio of soil-derived N, assuming that the ^{15}N /^{14}N ratio of reference plants correspond to that of plant available N in the soil.

The stable carbon isotope ratio (^{13}C /^{12}C) of plant material is depleted in the heavy stable carbon isotope (^{13}C) relative to that of CO_2 from the atmosphere, which is due to the discrimination against ^{13}C during diffusion of CO_2 through

the stomata and photosynthetic carbon assimilation. Variations in ^{13}C -discrimination have been used to differentiate between photosynthetic pathways in plants. In C_3-plant species, the ^{13}C -discrimination increases with an increasing ratio of intercellular and atmospheric partial pressures of CO_2. [20]. The ^{13}C /^{12}C ratio is affected by environmental conditions such as drought or nutrient availability. Therefore measurements of ^{13}C -discriminations can be used to screen for variation of water-use efficiency [21]. Consequently, the effect of changed environmental conditions such as inoculation or addition of organic material on water-use efficiency can be determined by measurements of the stable carbon isotope ratio (^{13}C /^{12}C) of plant material.

The aim of our study was to determine (i) whether inoculated *Frankia* strains remain active in the soil, i.e. determine the effect of storage of soil on the indigenous and the introduced *Frankia* population, (ii) the effect of the addition of dried alder leaves to the soil on the *Frankia* population, and (iii) through the use of ^{15}N /^{14}N signatures estimate the amount of soil NO_3^- which is taken up by the plants under the different treatments as well as the amount of fixed N_2. The $\delta^{15}N$ and $\delta^{13}C$ values of alder leaves, roots and soil were measured and *Frankia* populations in the root nodules were analyzed by *in situ* hybridization, where fluorescently labeled probes are used to identify groups of *Frankia* strains [22] [23].

4.3. Material and Methods

4.3.1. Bacteria strains

Frankia strains were grown for 4 weeks in P+N-medium [24] containing sodium propionate and ammonium chloride as C- and N-source, respectively. Cultures were harvested by centrifugation, washed twice in phosphate buffered saline (PBS, composed of 0.13 M NaCl, 7 mM Na_2HPO_4 and 3 mM NaH_2PO_4, pH 7.2 in water) [25] and homogenized in PBS by repeated passages through a needle (0.6 mm in diameter) with a sterile syringe [26].

4.3.2. Soil characterization, inoculation, and ^{15}N enrichment

Surface soil samples (to a depth of 20 cm) were collected from a sandy loam supporting a natural stand of *A. glutinosa* (Ettiswil, Switzerland) [23]. This soil was characterized by high nitrate concentrations (10 to 20 mM), a low content of organic material (0.02%) and the presence of *Frankia* subgroups IIIa, IIIb and IV of the *Alnus* host infection group. At the natural site, however, nodules were only formed by subgroup IIIa [23] [22]. Freshly sampled soil was cleared of larger particles, e.g. roots and stones, and sieved (mesh size 5 mm). Half of the soil was inoculated with a mixture of pure cultures of *Frankia* strains AgB1.9, Arl3, and Ag45/Mut15 each at an estimated density of 10^7 cells [g soil fresh wt.]$^{-1}$. The inoculated and the non-inoculated soils were then halved again. One half of each soil was mixed with ground dried alder leaves to a final concentration of 1%. The alder leaves were collected directly from alders at several natural alder stands (River Limmat, Switzerland) not identical with the site where the soil was harvested. Leaves were dried at 120°C for 3 days. The four soil sub-samples; (i) Control soil (C-soil); (ii) soil inoculated with *Frankia* pure cultures (F-soil); (iii) soil mixed with dried alder leaves (L-soil); and (iv) inoculated with *Frankia* pure cultures and mixed with dried alder leaves (FL-soil) were incubated in a climate chamber for 6 weeks (16h day/8 h night, 20°C/16°C) (= storage time) (Fig. 4.1).

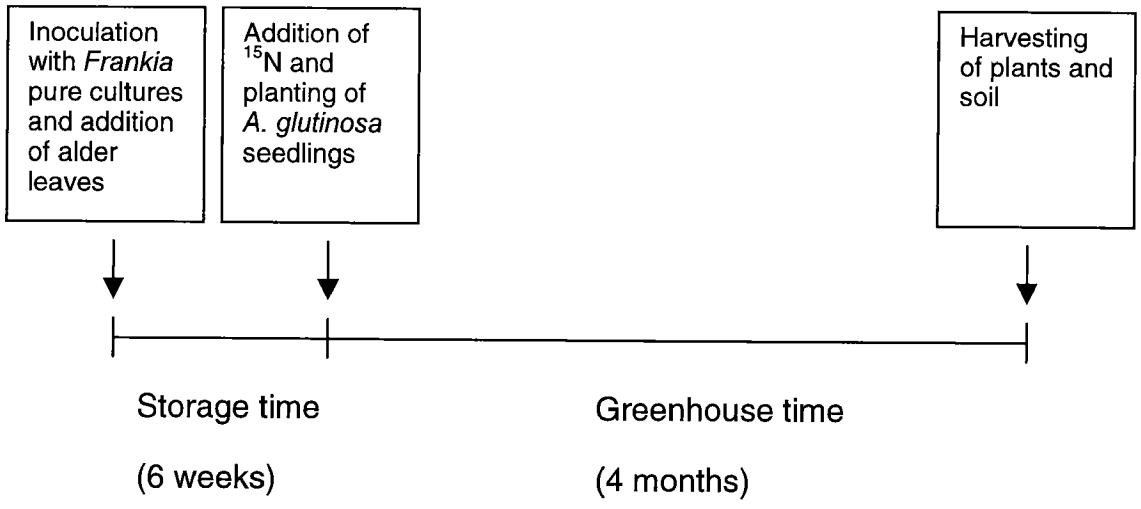


Figure 4.1. Different time periods in the course of the experiment.

After 6 weeks of storage, soil sub-samples (n=15, 800 g [fresh wt.]) from the four treated soils were mixed with 12 mg ^{15}N -$NaNO_3$ (Cambridge Isotope Laboratories, Andover, MA, USA; ^{15}N enrichment of 98%) and filled into 800 cm^3 pots. The addition of ^{15}N -NO_3^- resulted in about 3.76 at. % excess in available soil nitrogen (mathematically determined).

4.3.3. *Alnus glutinosa* growth conditions

Pots were planted with approximately 4-week-old seedlings of *A. glutinosa* (L.) Gaertn. that had been germinated and grown in Perlite supplemented with a modified Heller salt solution [27] containing 0.075 µM NO_3^- as nitrogen source at pH 5.4 [28] in a growth chamber with a thermoperiod of 24/18°C and a photo period of 16/8 h day/night. The pots were adjusted to and maintained at a matrix potential of -0.01 MPa. Plants were grown in the greenhouse with a thermoperiod of 28/22°C and a photoperiod of 16/8 h day/night for 4 months (December 14, 1998 to April 14, 1999) (= greenhouse time) (Fig. 4.1).

4.3.4. Analysis of soil parameters

Anion concentrations (NO_3^-, NO_2^-, SO_4^{2-}, PO_4^{3-}, and Cl^-) from pore water of soil samples collected (i) at the beginning of the study, (ii) after 2 weeks of storage, (iii) at planting time and (iv) at the end of the experiment were determined by ion chromatography (Dionex DX-100 ion chromatograph equipped with an IonPac AS4A-SC column; Dionex, Sunnyvale, USA) [29]. The pore water was obtained by centrifugation of approx. 12 g of soil filled into 5 ml plastic syringes which were plugged with silane treated glass wool (Supelco Inc., Bellefonte, USA). The filled syringes were placed in 15 ml Falcon tubes and centrifuged (2500 x g) for 10 minutes at 4°C. Pore water was collected and anion concentrations in pore water (µM) were correlated to water contents and expressed in µmol [g soil dry wt.]$^{-1}$.

4.3.5. Analysis of plant parameters

Plant height was monitored monthly. After 4 months of growth in the greenhouse, shoots, roots and nodules of the plants were harvested separately.

Shoots and roots were dried at 105°C for 24 h to determine dry weights and to calculate shoot/root mass ratios.

4.3.6. Stable isotope analysis

The dried plant samples were ground to a fine powder with a steel ball mill (Mixer Mill, Retsch MM2000, Germany). A continuous-flow mass spectrometer (DELTA-S Finnigan MAT, Bremen, Germany) was used to determine total nitrogen and carbon concentrations and isotope ratios (^{15}N /^{14}N and ^{13}C /^{12}C) of the aliquots. The samples, usually 1 mg, were combusted with an elemental analyzer (EA-1110, Carlo Erba, Rodano, Italy) which was connected to the mass spectrometer via an interface (CONFLO II Finnigan MAT, Bremen, Germany). Both carbon and nitrogen isotope ratios were measured from the same sample in a single run. The isotopic values of the samples were expressed in the delta-notation relative to the international standard for nitrogen (atmospheric N_2) or carbon (PeeDee Belemnite, PDB):

$$\delta^{15}\mathrm{N\ or\ }\delta^{13}\mathrm{C}\ (‰) = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] * 10^3 \qquad (1)$$

where R is the isotope ratio of the sample and standard. The working standards for $\delta^{15}N$ and $\delta^{13}C$ were calibrated with standards supplied by the IAEA (Vienna, Austria). For the correction of instrumental drifts, plant material as working standard of known nitrogen and carbon concentrations and isotopic compositions was used each time 10 samples were analyzed. The standard deviation for replicate analysis of a leaf standard material was 0.3‰. The nitrogen content was determined from the total peak area of the mass spectrometer signals for the masses 28 and 29 (for N_2) (rel. standard deviation =1%) and the carbon content from the signals of the masses 44, 45 and 46 (for CO_2).

All nitrogen isotope data in this ^{15}N -enrichment study were expressed as atom % excess (at%) in ^{15}N, which was calculated from measured $\delta^{15}N$ values:

$$\text{Atom \% excess}_{\text{sample}} = \left\{ \frac{\left({}^{15}\text{N} / {}^{14}\text{N}_{\text{sample}} \right)}{1} + \left({}^{15}\text{N} / {}^{14}\text{N}_{\text{sample}} \right) \right\} * 100 - 0.3663 \quad (2)$$

The N_2-fixation rates of alders grown on either C-, L-, F-, or FL-soils were estimated by the isotope dilution method [30]. The ^{15}N /^{14}N ratio of soil derived N was obtained from at. % excess in ^{15}N values of a "non-N_2-fixing" reference alder that accumulated N predominantly from soil, relative to their N_2-fixing counterparts. In this way, the different treatments could be ranked for fixation ability:

$$N_2 \text{ fixed} / N \text{ total} = 1 - \frac{(\text{at\% excess})_{\text{sample}}}{(\text{at\%excess})_{\text{control}}} \quad (3)$$

4.3.7. Analysis of *Frankia* populations in root nodules

For the *in situ*-hybridization analysis of *Frankia* populations in root nodules, all nodules were harvested. Nodule lobes were counted and fresh weights of nodules determined. Nodules were split into lobes and fixed in 4% paraformaldehyde in PBS at 4°C for 16 h [31]. Nodule lobes were subsequently washed in PBS and ground in a mortar. Lobes were stored in 96% ethanol at -20°C [31]. Samples (3µl) of lobe homogenates were air-dried on gelatin-coated slides (0.1% gelatin, 0.01% $KCr(SO_4)_2$) for at least 2 h [32]. After dehydration in 50, 80 and 96% ethanol for 3 min each, the preparations were pretreated with SDS/DTT (10 mg ml^{-1} SDS, 50 mM dithiothreitol (DTT, Fluka) in water, freshly prepared) at 65°C for 30 min followed by an incubation with lysozyme, 37 320 U dissolved in 1 ml of 100 mM Tris/HCl, pH 7.5, 5 mM EDTA (Fluka, Buchs, Switzerland,) at 37°C for 10 min [23]. Afterwards, the samples were rinsed with distilled water and dehydrated as described above.

Oligonucleotide probes targeting 16S rRNA of the domain Bacteria (Eub338) [33] or specific sequences on the 23S rRNA insertion of *Frankia* strains AgB1.9 (probe B1.9) [23], Arl3 (probe 23Arl3) [23] and Ag45/Mut15 (probe 23Mut(II)) [23] representing *Alnus* host infection groups I, IIIa, and IV, respectively [34]

were synthesized with a primary amino group at the 5' end (C6-TFA, MWG, Ebersberg, Germany). The fluorescent dye Cy3 (Amersham, Zurich, Switzerland) was covalently bound to the amino group of the oligonucleotide probe. The dye-oligonucleotide conjugate (1:1) was purified from unreacted components and stored at -20°C in distilled water at a concentration of 25 ng μl^{-1} [33]. This solution was amended with the DNA specific dye 4'6-diamidino-2-phenylindole (DAPI) (Sigma, Buchs, Switzerland) to give a final concentration of 10 $\mu g\ ml^{-1}$.

Hybridizations were performed in 9 µl of hybridization buffer (900 mM NaCl, 20 mM Tris/HCl, 0.01% SDS, pH 7.2) in the presence of 30% formamide [35] and 1 µl of oligonucleotide probe (25 ng) at 42°C for 2 h [23]. After hybridization, the slides were washed in hybridization buffer without formamide at 48°C for 20 min, rinsed with distilled water, and air-dried. Preparations were mounted with Citifluor solution (Citifluor, Canterbury, UK) and examined with a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) fitted for epifluorescence detection with a high-pressure mercury bulb (50 W). DAPI-stained bacteria were analyzed with filter set 02 (Zeiss; G365, FT395, LP420), whereas binding of Cy3-labeled probes was detected with filter set HQ-Cy3 (AHF Analysentechnik, Tübingen, Germany; G535/50, FT565, BP610/75).

4.3.8. Statistic analysis

All data were expressed as mean ± standard deviation and assessed by multiple pairwise comparisons with Tukeys HSD test (SYSTAT). The significance level was set at $p<0.05$.

4.4. Results

4.4.1. Soil parameters

The NO_3^- concentrations of the pore water changed significantly during the 6 weeks of storage. Within 2 weeks, the initially high NO_3^- concentration (10 mmol [g soil dry wt.]$^{-1}$ decreased to a concentration below the detection limit (1 µM) in L- and FL-soil, which were both mixed with dried alder leaves. After 6

weeks of storage, the NO_3^- concentration in those soils increased again to the initial concentration of 15 mmol [g soil dry wt.]$^{-1}$. Concentration of NO_3^- in F- and C-soils, both incubated without leaves, remained high during the storage time.

At the end of the experiment (i.e. when plants were harvested) no significant differences between the treatments could be detected regarding water content, pH, concentrations of SO_4^{2-}, Cl^-, PO_4^{3-}, NO_3^- and NO_2^- in the pore water, and $\delta^{15}N$ values of bulk soil. However, concentrations of NO_3^- in soil from single pots varied from 0.5 mmol [g soil dry wt.]$^{-1}$ to 75.4 mmol [g soil dry wt.]$^{-1}$.

When plants were harvested, the water content of soils was 16±7.7%. The pH of the pore water was 7.1 when soils were harvested and no major changes in pH were observed throughout the duration of the experiment. In all soil sub-samples SO_4^{2-} concentrations (0.1±0.2 mmol [g soil dry wt.]$^{-1}$) and Cl^- concentrations (0.7±1.3 mmol [g soil dry wt.]$^{-1}$) remained in the same range throughout the whole experiment. PO_4^{3-} concentrations were always below the detection limit at 20 µM.

The NO_2^- concentrations, which had been below the detection limit (1 µM) both during storage and at the beginning of greenhouse time, increased to 0.1±0.2 mmol [g soil dry wt.]$^{-1}$ during greenhouse time, which showed microbial activities of denitrification.

The at. % excess in ^{15}N values of soils at the end of the experiment were between 0.27±0.19 and 0.58±0.81 and not significantly different. The carbon contents of L, F-, and FL-soils (5.1±0.3%), were not significantly different, while the control soil had a lower carbon content, measuring 4.8±0.3%. On the other hand, there were significant differences in the nitrogen content in soils incubated with leaves as compared to soils incubated without leaves. At the end of the experiment, soils which had been incubated with leaves had a nitrogen content of 0.14±0.01 % whereas those without leaves had a nitrogen content of only 0.12±0.01%.

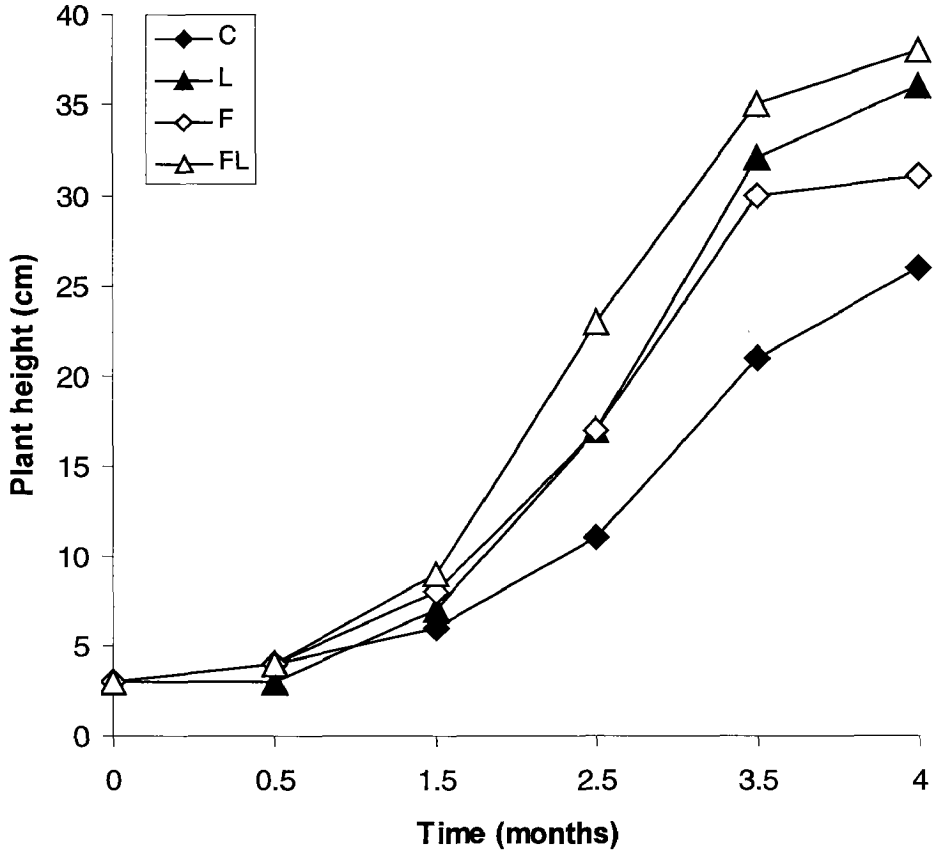
4.4.2. Plant parameters

Leaves of control plants were slightly chlorotic, in contrast, leaves of the other plants from L-, F-, and FL-soil were dark green.

Monthly plant height measurements indicated a faster growth of inoculated plants compared to non-inoculated plants. Similarly, plants on soil with leaves grew faster compared to plants on soil without leaf amendment (Fig. 4.2). After 4 months of growth, alders on C-soil were the smallest plants measuring an average of 26±7 cm high, whereas alders on FL-soil were the largest plants measuring 38±7 cm high. Plants on L-soil were taller than plants on F-soil (Table 4.1). No significant differences were found between plants on soils which had been inoculated with *Frankia* cultures and their non-inoculated counterparts. However, plants on soils that had been incubated with leaves were significantly taller than plants from soils incubated without leaves. A pattern similar to that of plant height measurements was found for plant dry weights (Table 4.1). The lowest dry weights were obtained from plants grown on C-soil (3±1.3 g [dry wt.]), and the highest ones from FL-soil plants (5.7±1.6 g [dry wt.]). Plants from soils incubated with either dried leaves or *Frankia* pure cultures were 4.3±1.7 and 4.1±2.5 g [dry wt.], respectively. Again, higher values were determined for plants which were grown on soils mixed with leaves.

The root/shoot ratios of plants from the different treatments were in the range of 0.7±0.2 to 0.8±0.2 and were not significantly different (Table 4.1). The carbon concentration of leaves was similar in all treatments (47±1%) with the exception of leaves from C-soil-plants, which had a slightly lower carbon concentration (46±1%). The nitrogen concentration of leaves was higher in non-inoculated plants (3.2±0.3% and 3.3±0.3%) than in inoculated plants (3±0.3% for both treatments). Consequently, C/N ratios of leaves from plants grown on inoculated soils were higher (16±1 for both treatments) than C/N ratios of leaves from plants grown on non-inoculated soils (15±1 for both treatments). The carbon content of roots was slightly higher in plants grown on inoculated than on non-inoculated soil (Table 4.1). Roots from plants grown on soil incubated with leaves had a significantly higher nitrogen concentration (2.2±0.3 and 2.4±0.3%) compared to plants from soils without leaves (2±0.2 and 2.1±0.2), with slightly higher nitrogen contents in the roots of inoculated plants.

Consequently C/N ratios for roots were the highest for control plants (21±3) and the lowest for plants grown on inoculated soil with leaves (18±3). Values for plants grown on soil with either *Frankia* or leaves were intermediate when compared to C/N ratios of the control plants and of plants grown on FL-soil.



Plant height (cm)	14 Dec	23 Dec	23 Jan	23 Feb	23 March	14 April
C	3 ± 0.5	4 ± 1	6 ± 2	11 ± 4	21 ± 6	26 ± 7
L	3 ± 0.5	3 ± 1	7 ± 2	17 ± 5	32 ± 8	36 ± 10
F	3 ± 0.5	4 ± 1	8 ± 4	17 ± 9	30 ± 7	31 ± 9
FL	3 ± 0.5	4 ± 1	9 ± 3	23 ± 6	35 ± 7	38 ± 7

Figure 4.2. Height of *A. glutinosa* seedlings grown on control soil (C), inoculated soil (F), soil mixed with alder leaves (L) or inoculated soil mixed with alder leaves (FL).

Table 4.1. Plant parameters after 4 months of growth

Plant parameters	Soils without leaves[a]		Soils with leaves[c]	
	Non-inoculated[a] C	Inoculated[b] F	Non-inoculated[a,c] L	Inoculated[b,c] FL
Plant height (cm)	26 ± 7 [d]	30 ± 9 [d,e]	36 ± 10 [e,f]	38 ± 7 [f]
Plant weight (g [dry wt.])	2.9 ± 1.3 [d]	4.1 ± 2.5 [d,e,f]	4.3 ± 1.7 [e]	5.6 ± 1.6 [f]
Leaves (g [dry wt.])	0.4 ± 0.2 [d]	0.6 ± 0.3 [d,e]	0.6 ± 0.3 [e]	0.8 ± 0.2 [e]
Stem (g [dry wt.])	1.2 ± 0.5 [d]	1.7 ± 1.0 [d,e]	1.9 ± 0.9 [e,f]	2.4 ± 0.7 [f]
Roots (g [dry wt.])	1.3 ± 0.6 [d]	1.8 ± 1.2 [d,e]	1.7 ± 0.8 [d,e]	2.4 ± 0.8 [e]
Root/shoot ratio	0.8 ± 0.2 [d]	0.7 ± 0.2 [d]	0.7 ± 0.3 [d]	0.8 ± 0.2 [d]
C concentration of leaves (%)	46 ± 0.8 [d]	47 ± 1.2 [d,e]	47 ± 0.9 [e]	47 ± 0.8 [e]
N concentration of leaves (%)	3.2 ± 0.3 [d,e]	3.0 ± 0.3 [e]	3.3 ± 0.3 [d]	3.0 ± 0.3 [e]
C/N ratio of leaves	15 ± 1.4 [d]	16 ± 1.3 [e]	14 ± 1.4 [d]	16 ± 1.4 [e]
C concentration of roots (%)	42 ± 1.3 [d]	43 ± 1.4 [d]	42 ± 1.7 [d]	44 ± 1.3
N concentration of roots (%)	2.0 ± 0.2 [d]	2.1 ± 0.2 [d,e]	2.2 ± 0.3 [e,f]	2.4 ± 0.3 [f]
C/N ratio of roots	21 ± 3.1 [d]	20 ± 2.3 [d,e]	19 ± 2.3 [d]	19 ± 3.0 [d]

[a] soil "Ettiswil"

[b] soil "Ettiswil", inoculated with *Frankia* strains Arl3, Ag45/Mut15 and AgB1.9 at a density of each approx. 10^7 cells [g soil fresh wt.]$^{-1}$

[c] soil "Ettiswil" mixed with dried alder leaves to a final concentration of 1%

[d,e,f] values with the same character are not significantly different ($p \leq 0.05$)

4.4.3. Stable isotopes

The at. % excess in ^{15}N values of the total plant material (pooling root, stem and leaves) from the different treatments were used in equation (3) to estimate the percentage of plant nitrogen which was fixed. This equation used the values of at. % excess in ^{15}N of a poorly N_2-fixing reference alder in which only two nodules (total nodule weight 10 mg) were detected. The at. % excess values in

^{15}N of roots were always higher than those of stems, and those of stems were always higher

Table 4.2. Stable isotope parameters

Stable isotope parameters	Soils without leaves[a]		Soils with leaves[c]	
	Non-inoculated[a]	Inoculated[b]	Non-inoculated[a,c]	Inoculated[b,c]
	C	F	L	FL
At. % excess of leaves	1.1 ± 0.6[d]	0.9 ± 0.9[d,e]	0.7 ± 0.3[e]	0.6 ± 0.5[e]
At. % excess of stems	1.6 ± 0.8[d]	1.2 ± 0.8[d,e]	1.1 ± 0.5[d,e]	0.8 ± 0.6[e]
At. % excess of roots	2.0 ± 1.0[d]	1.4 ± 0.8[d,e]	1.3 ± 0.7[e]	1.3 ± 0.9[e]
Amount of fixed nitrogen (%)	33 ± 19	53 ± 34[d]	58 ± 25[d]	78 ± 24
Soil-N uptake (%)	6.1 ± 2.2[d]	5.9 ± 3.7[d]	6.0 ± 4.0[d]	7.1 ± 4.7[d]
$\delta^{13}C$ values of leaves	-30.5 ± 0.8[d]	-29.7 ± 1.6[d,e]	-29.8 ± 0.8[e]	-29.2 ± 1.1[e]
$\delta^{13}C$ values of roots	-30.1 ± 0.7[d]	-29.5 ± 1.8[d,e]	-29.3 ± 1.2[e]	-28.7 ± 1.3[e]

[a] soil "Ettiswil"

[b] soil "Ettiswil", inoculated with *Frankia* strains Arl3, Ag45/Mut15 and AgB1.9 at a density of each approx. 10^7 cells [g soil fresh wt.]$^{-1}$

[c] soil "Ettiswil" mixed with dried alder leaves to a final concentration of 1%

[d,e] values with the same character are not significantly different ($p \leq 0.05$)

than those of leaves. The at. % excess in ^{15}N of leaves, roots, and stems from plants grown on inoculated soils (F- and FL-soils) were lower than those in plants grown on non-inoculated soils (C- and L-soils). The same was true for plants grown on soils mixed with leaves (L- and FL-soil) compared to plants grown on soil without leaves (C- and F-soil) (Table 4.2). The highest values were always detected in plants from C-soil as compared to the lowest values in plants from FL-soil. The fertilizer uptake in all treatments, calculated according to equation (3), was 5.9 to 7.1% and not significantly different. The percentage

of plant nitrogen fixed by *Frankia* increased in the order of C-, L-, F- and FL-soils (Table 4.2).

The patterns of the plant heights correlated with $\delta^{13}C$ values of plant material. Plant size directly correlated with ^{13}C -depletions in plant biomass as the $\delta^{13}C$ values of plants growing on C-soil were significantly depleted relative to the values of plants on FL-soil (Table 4.2).

4.4.4. *Frankia* populations in root nodules

The total weight of nodule lobes per plant was not significantly different in all treatments although the number and size of lobes differed (Table 4.3). The number of nodule lobes formed on plants grown on inoculated soil was higher than on plants which had been grown on non-inoculated soil. In contrast the biggest nodules could be found on non-inoculated plants and lobes from plants grown on inoculated soil were significantly smaller. On non-inoculated soils there was no significant difference between the leaf-treated soil and the non-treated soil regarding lobe number and size (Table 4.3). However, on inoculated soils a significant difference in the number of nodule lobes was found between the leaf-treated soil and the untreated soil.

Almost all nodule lobes contained nitrogen-fixing *Frankia* populations except for 7-17% of the nodule lobes which did not contain filaments, vesicles or spores typical for frankiae. There were no significant differences concerning the number of empty nodule lobes between the treatments. In nodules from plants grown on inoculated soils *Frankia* strains belonging to subgroup IV and IIIa were both identified with probes 23Mut(II) and 23Arl3, respectively. Nodule lobes on plants grown on inoculated soil without leaves contained high percentages of *Frankia* from both subgroups, IV (51 ± 20%) and IIIa (32±23%). In contrast plants grown on inoculated soil treated with leaves had significantly less nodules infected by subgroup IIIa (8±6) than by subgroup IV (81±11).

Table 3 Nodulation parameters

Nodulation parameters	Soils without leaves[a]		Soils with leaves[c]	
	Non-inoculated[a]	Inoculated[b]	Non-inoculated[a]	Inoculated[b]
	C	F	L	FL
Number of lobes	21 ± 13[d]	31 ± 15 [d]	20 ± 13 [d]	80 ± 28
Total lobe weight (mg [fresh wt.])	160 ± 76 [d]	149 ± 82 [d]	169 ± 68 [d]	165 ± 42 [d]
Average lobe weight (mg [fresh wt.])	9 ± 5 [d]	5 ± 1	10 ± 4 [d]	2 ± 1
IIIa (%)	93 ± 16	32 ± 23	0	8 ± 6
IV (%)	0	51 ± 20	89 ± 12	81 ± 11
I (%)	0 [d]	0 [d]	0 [d]	0 [d]
Lobes without *Frankia* (%)	7 ± 16 [d]	17 ± 14 [d]	11 ± 12 [d]	11 ± 13 [d]

[a] soil "Ettiswil"

[b] soil "Ettiswil", inoculated with *Frankia* strains Arl3, Ag45/Mut15 and AgB1.9 at a density of each approx. 10^7 cells [g soil fresh wt.]$^{-1}$

[c] soil "Ettiswil" mixed with dried alder leaves to a final concentration of 1%

[d] values with the same character are not significantly different ($p \leq 0.05$)

Frankia in lobes from plants grown on non-inoculated soil without leaves were all detectable with probe 23Arl3 (93±16%). None of the other *Frankia*-specific probes (23B1.9, and 23Mut(II)) showed hybridization signals. However, *Frankia* in lobes from plants grown on non-inoculated soil with leaves were all detectable with probe 23Mut(II) (89±12%).

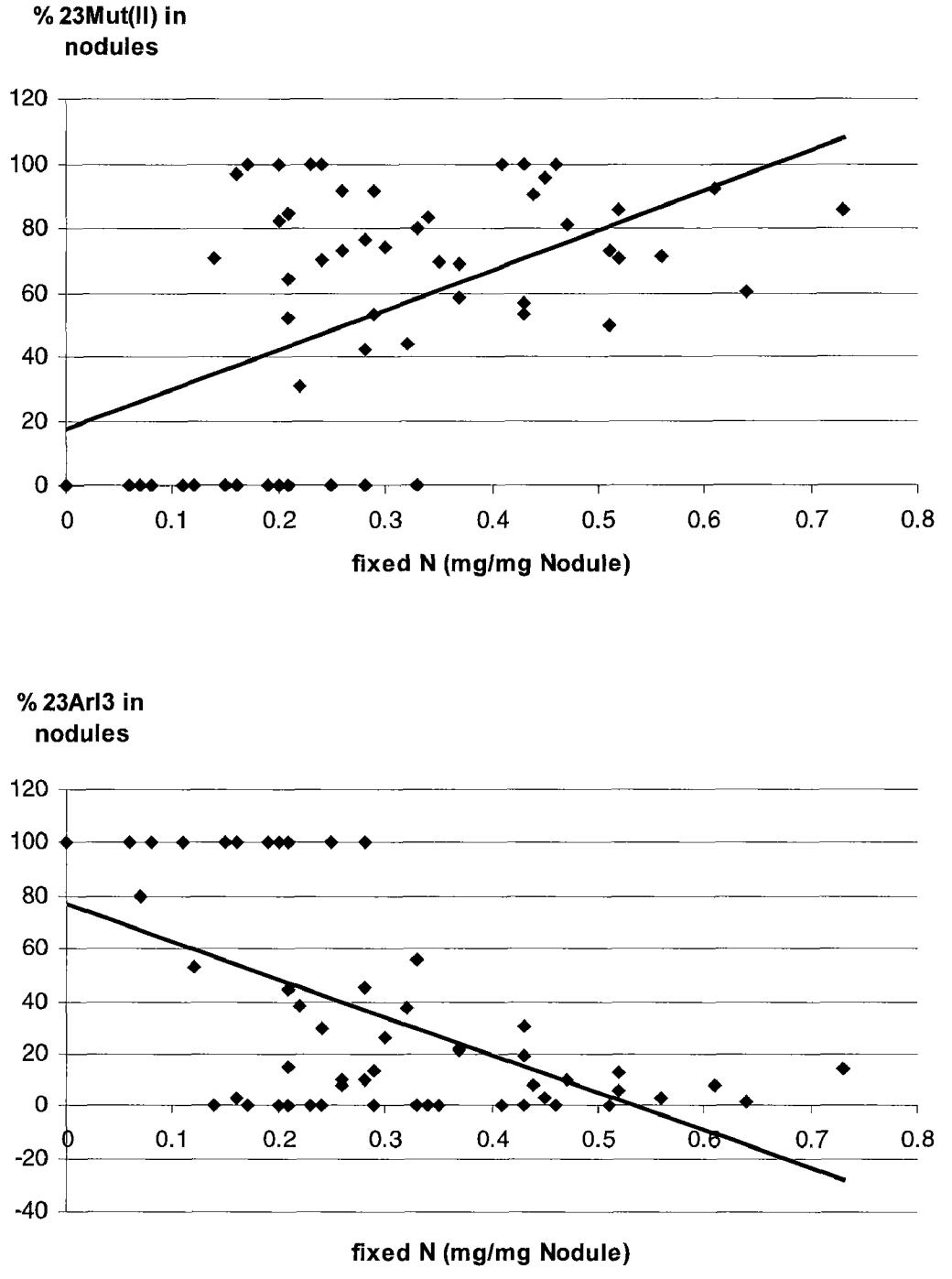


Figure 4.3. Correlation between N_2-fixing rate and *Frankia* population in the nodules

A correlation between N_2-fixing rate and the *Frankia* population in the nodules could be observed. There was a significant difference between the two *Alnus* host infection groups regarding N_2-fixing rate. The percentage of *Alnus* host infection group IV was positively, the percentage of *Alnus* host infection group IIIa negatively correlated with the N_2-fixing rate (Fig. 4.3) with a gradient of 0.0021 ± 0.002 and (-0.0023) ± (-0.002), respectively.

4.5. Discussion

4.5.1. Soil nutrients and nitrogen availability

The purpose of this study was to reveal how the structure and N_2-fixing activity of a *Frankia* population would be affected by a 6-weeks storage time after i) inoculation with *Frankia* pure cultures and ii) addition of dried alder leaves. Since the construction of the experiment was based on different pretreatments of the soil prior to seedling planting, soil parameters such as water content, pH and concentration of anions were to remain constant. This was achieved, although soils which had been mixed with dried alder leaves had a higher nitrogen content. Plant growth of *A. glutinosa* could therefore also be promoted by the availability of different sources of nitrogen, although the measured $\delta^{15}N$ values indicated plant growth promotion by N_2-fixation.

In soils supplemented with alder leaves, the NO_3^- concentration decreased during the storage time in the climate chamber to values below the detection limit and increased within 6 weeks to values comparable to those at the beginning of the experiment. This is in contrast to soils receiving no treatment of alder leaves. We suggest that NO_3^- was first used for the mineralization of the alder leaves and later released from dead microorganisms. High NO_3^- concentrations can influence the nodulation capacity [36], although another study reported that nodulation was not affected by soil nitrogen [37]. The development of the *Frankia* populations in the soil and thus nodulation capacity might therefore not be influenced by substances in the leaves exclusively, but

also by different redox conditions during storage time, the availability of O_2 and the availability of NO_3^- as a source of nitrogen.

4.5.2. Plant growth stimulation

Since it is known that inoculation with *Frankia* strains promotes plant growth [14] [13], we expected taller plants to grow on inoculated soils. However, the effect of storage on the inoculated *Frankia* pure cultures was unknown. Our results show that besides the inoculation, which stimulated plant growth although seedlings were planted 6 weeks after inoculation, the addition of dried alder leaves significantly enhanced the growth of plants compared to the control plants. It was shown that alder seeds contain substances which enhance nodulation [17]. In our experiment, better growth rates were not achieved through a higher nodulation rate but, as $\delta^{15}N$ measurements indicated, by a higher N_2-fixation. The higher N_2-fixation was possibly caused by an activation of the *Frankia* population, in particular by the activation of a strain of *Alnus* host infection group IV, since the N_2-fixation rate was positively correlated with the presence of this strain in the nodules. We assume that the slower growth of larger plants towards the end of the experiment was caused by space limitations in the relatively small pots. Plants grown on inoculated soil with leaf treatment were the tallest and had the greatest dry biomass. However, when compared to the positive effect of inoculation at the time of planting [12] the positive effect of inoculation of the soil 6 weeks before planting was smaller.

4.5.3. Nitrogen uptake and water use efficiency

Leaves of control plants were slightly chlorotic, indicating nitrogen deficiencies. In contrast, leaves of the other plants from L-, F-, and FL-soil were dark green. The results from visual observations were confirmed by the stable isotope measurements. Significantly lower at. % excess in ^{15}N values of roots, stems, and leaves were measured in plants from pretreated soils, relative to those of control plants. The lower at. % excess in ^{15}N values of plant material clearly indicates higher activities of *Frankia* fixing unlabeled N_2. This higher N_2-fixing

activity resulted in better growth of alders in the treated soils. On the other hand, significantly different at. % excess in ^{15}N values between alder roots and leaves were determined. Root values were always higher values than leaf values, indicating that the labeled soil-NO_3^- was preferentially used in the roots [38, 39]. Using the at. % excess in ^{15}N values of total plant material, it was possible to estimate the relative contribution of N from Frankia to total N-plant by the ^{15}N isotope dilution method [19]. The results showed that the relative N_2-fixation rate of alder was only 33% in the C-soil, which was in contrast to a 53% and 58% rate for alder from F- or L-soil, respectively, and 78% when alder was grown in FL-soil. The latter N_2-fixation rate was similar to what is usually found for alder (70-90%) [2] [3]. Kurdali *et al.* [40] showed that the N_2-fixation rate of alder was 74% using the ^{15}N isotope dilution technique. It is possible, that the N_2-fixation rates are slightly underestimated in our study since the reference plant had two nodules containing *Frankia*, which may have fixed small amounts of N_2. In the present study, the higher N_2-fixing activity of larger plants was correlated with high nitrogen content in their roots. However, a higher nitrogen fixing activity did not lead to a higher nitrogen content in the leaves. In the field, nitrogen concentrations in alder leaves are in the range of 2-3% [11] which is in agreement with our results.

The results from the nitrogen isotope data of alder were in good agreement with variations in $\delta^{13}C$ values in the plant material. In the literature, carbon isotope discriminations of plants are usually negatively correlated with their water-use-efficiency at the whole plant level [41, 42]. The correlation reported in the literature corresponded to the $\delta^{13}C$ value of alder leaves and roots measured in the present study. Here, alder leaves and roots from L-, F, and FL-soil (high N_2-fixation rates) were significantly ^{13}C -enriched (more positive $\delta^{13}C$), relative to the plant material from C-soil (low N_2-fixation rates). This means that a lower ^{13}C -discrimination rate was determined for alder with high N_2-fixation rates, indicating improved water use efficiency. However, other N_2-fixing plants such as legumes or *Casuarina* were assumed to be less water-use efficient than plants utilizing soil mineral N, because of the high respiratory requirements for

driving N_2-fixation. In these studies, the lowest ^{13}C -enrichments were obtained for N_2-fixing plants relative to those grown on NO_3^- and NH_4^+ indicating a lower water-use efficiency of plants with high N_2-fixation rates. This effect, a lower water-use-efficiency of alders with high N_2-fixation rates was not found in the present study.

4.5.4. *Frankia* populations in root nodules

The infectious capacity of a soil depends on the environmental conditions [43] [44] as well as on the activity of the *Frankia* population. The population in the nodules does not represent the total population in the soil but only the fraction of physiologically active, infective frankiae [15]. Therefore the activation of the indigenous population by the addition of certain substances into the soil could have a positive effect on plant growth. This could be shown by the addition of alder leaves to the soil prior to planting. Lobes from plants grown on control soil contained only a strain belonging to subgroup IIIa of the *Alnus* host infection group, although it is known that the indigenous *Frankia* population in the soil used in this study consists of subgroups IIIa, IIIb and IV of the *Alnus* host infection group [22]. On the same soil with leaves added, all lobes were infected by the native strain belonging to subgroup IV of the *Alnus* host infection group. We postulate that this strain was either activated by substances in the leaves as is the case for *Casuarina* infective strains, which are promoted by *Casuarina* leaves [16] or it was promoted by the higher N content of the soil or the short time during storage when the NO_3^- concentration was extremely low. Since the doubling time of strain Arl3 was reduced by some compounds in alder seeds [45] growth of the strain belonging to subgroup IIIa of the *Alnus* host infection group could also have been hindered by the addition of the leaves, the higher soil N content or the low NO_3^- content. Because of these conditions it could be outcompeted by the native strain belonging to subgroup IV of the *Alnus* host infection group. It is not known whether *Frankia* can grow saprophytically in soils without host plants but since active *Frankia* can be found in soil devoid of host plants [46] [47] [43] [48] it is assumed that they can at least survive outside

the nodule. This is confirmed by Zimpfer et al. [46] who showed that dry storage of a soil for 3.5 years did not change its infectious capacity.

Inoculation with the two nitrogen-fixing *Frankia* strains Arl3 and Ag45/Mut15 demonstrated that both outcompeted the indigenous *Frankia* populations displaying comparable competitive abilities in soils without leaves. The addition of leaves had the same effect as on non-inoculated soils. A strain belonging to subgroup IV of the *Alnus* host infection group could be detected in almost all nodules and largely outcompeted strains belonging to subgroup IIIa. One possible reason for the enhanced competitive fitness of strain Ag45/Mut15 is that Ag45/Mut15 grows faster than Arl3 at room temperature [49]. Since the soil was stored between 16°C and 20°C, storage temperature could have impacted the competition between strains. Nevertheless, it is surprising that the addition of alder leaves to a soil that was harvested beneath the canopy of a natural alder stand has such an enormous effect on the indigenous *Frankia* population. Leaves were added before the seedlings were planted and it has been speculated that the competition pressure is lower in soils without host trees [50]. Other possible reasons might be that the added leaves were not derived from the trees growing at the same site from which the soil was harvested, thus their chemical composition might have been slightly different. Additionally, the nitrate concentration in soils with leaves decreased to very low values during the incubation time.

Through whole cell *in situ* hybridization and isotope analysis, bacterial identity was directly linked with bacterial activity. The structure of the Frankia population was quantified by *in situ* hybridization and their N_2-fixation was quantified by measurements of ^{15}N enrichment in plant material. The combination of both methods has a great potential to be applied at field sites. In the present study it was used to identify the key bacterial members involved in nitrogen fluxes and for in situ qualifications and quantifications of key processes. The application of the $\delta^{13}C$ values provides long-term information concerning the influence of the nitrogen source on the gas exchange characteristics and water balance of the plant.

4.6. Conclusion

In summary these results show that: 1. Introduced *Frankia* strains can compete for nodule formation with the indigenous population; even if the inoculation took place weeks before the seedlings were planted. 2. Inoculation as well as the addition of dried alder leaves promotes N_2-fixing activity and therefore plant growth; even in soils with high NO_3^- contents. 3. The addition of dried alder leaves promotes a *Frankia* strain belonging to subgroup IV under the applied conditions.

Further studies, however, need to address long-term effects of such an inoculation on nodulation activity. The potential activation of indigenous *Frankia* populations through increasing the availability of nutrients rather than through inoculation with pure cultures warrants investigation as well.

4.7. References

1. **Dommergues, Y.R.** 1996. La fixation d'azote chez les plantes actinorhiziennes et ses applications. Acta botanica Gallica 143(7): 663-679.

2. **Dommergues, Y.R.** 1996. Contribution of actinorhizal plants to tropical soil productivity and rehabilitation. Soil Biology and Biochemistry 29(5/6): 931-941.

3. **Huss-Danell, K.** 1997. Actinorhizal symbioses and their N_2-fixation. New Phytologist 136: 375-405.

4. **Huss-Danell, K.** 1986. Growth and production of leaf litter nitrogen by *Alnus incana* in response to liming and fertilization on degenerated forest soil. Canadian Journal of Forest Research 16: 847-853.

5. **Dawson, J.O. and J.W. Van Sambeck**. 1993. Interplanting woody nurse crops promotes differential growth of black walnut saplings. In: Proceedings, 9th Central Hardwood Forest Conference, Eds.: A.R. Gillespie, P.E. Pope, G. Rink, U.S. Department of Agriculture, Forest Service, North Central Forest Experiment Station, West Lafayette: 455-463.

6. **Moiroud, A.** 1996. Diversité et écologie des plantes actinorhiziennes. Acta Botanica Gallica 143(7): 651-661.

7. **Buresti, E., A.M. Domenach, M. Bosco and A. Moiroud**. 1990. Comparision between *Quercus robur/Alnus cordata* mixed plantation and *Quercus robur* in monoculture. In: Fifth International Symposium on Nitrogen Fixation with Non-Legumes. Kluwer Academic Publishers. Florence, Italy.

8. **Davey, C.B. and A.G.W. II**. 1984. Nitrogen fixation systems in forest plantations. In: Nutrition of plantation forests. Academic Press London, London: 361-377.

9. **Chow, P.** 1998. Stem and branch chemical composition of juvenile *Eleagnus* and *Alnus* species. In: 11.th International Frankia and

Actinorhizal Plant Conference. University of Illinois at Urbana-Champaign.

10. **Mikola, P.** 1975. Afforestation of bogs after industrial exploitation of peat. Silva Fenn 9: 101-115.

11. **Huss-Danell, K. and H. Ohlsson**. 1992. Distribution of biomass and nitrogen among plant parts and soil nitrogen in a young *Alnus incana* stand. Canadian Journal of Botany 70: 1545-1549.

12. **Nickel, A., D. Hahn, K. Zepp and J. Zeyer**. 1999. *In situ* analysis of introduced *Frankia* populations in root nodules of *Alnus glutinosa* grown under different water availability. Canadian Journal of Botany 77(9): 1231-1238.

13. **Steele, D.B., K. Ramirez and M.D. Stowers**. 1989. Host plant growth response to inoculation with *Frankia*. Plant and Soil 118: 139-143.

14. **Strukova, S., M. Vosatka and J. Pokorny**. 1996. Root symbioses of *Alnus glutinosa* (L.) Gaertn. and their possible role in alder decline: a preliminary study. Folia Geobot. Phytotax. 31: 153-162.

15. **Maunuksela, L., K. Zepp, T. Koivula, J. Zeyer, K. Haahtela and D. Hahn**. 1999. Analysis of *Frankia* in three soils devoid of actinorhizal plants. FEMS Microbiology Ecology 28: 11-21.

16. **Zimpfer, J.F., G.J. Kennedy, C.A. Smyth, J. Hamelin, E. Navarro and J.O. Dawson**. 1998. Localization of *Casuarina*-infective *Frankia* near *Casuarina cunninghamiana* trees in Jamaica. Canadian Journal of Botany 77 (9): 1248-1256.

17. **Benoit, L.F. and A.M. Berry**. 1997. Flavonoid-like compounds from seeds of red alder (*Alnus rubra*) influence host nodulation by *Frankia* (Actinomycetales). Physiologia Plantarum 99: 588-593.

18. **Danso, S.K.A.** 1995. Assessment of biological nitrogen fixation. Fertilizer Research 42(1/3): 33-41.

19. **Fried, M. and V. Middelboe**. 1977. Measurement of amount of nitrogen fixed by a legume crop (Soybean). Plant and Soil 47(3): 713-715.

20. **Farquhar, G.D., K.T. Hubick, A.G. Condon and R.A. Richards**. 1989. Carbon isotope fractionation and plant water-use efficiency. In: Stable isotopes in ecological research, Eds.: P.W. Rundel, J.R. Ehleringer, and K.A. Nagy, Springer, New York.

21. **Hubick, K.T., G.D. Farquhar and R. Shorter**. 1986. Correlation between water-use efficiency and carbon isotope discrimination in diverse peanut (Arachis) germplasm. Australian Journal of Plant Physiology 13(6): 803-816.

22. **Zepp, K., D. Hahn and J. Zeyer**. 1997. *In situ* analysis of introduced and indigenous *Frankia* populations in soil and root nodules obtained on *Alnus glutinosa*. Soil Biology and Biochemistry 29(9/10): 1595-1600.

23. **Zepp, K., D. Hahn and J. Zeyer**. 1997. Evaluation of a 23S rRNA insertion as target for the analysis of uncultured *Frankia* populations in root nodules of alders by whole cell hybridization. Systematic and Applied Microbiology 20: 124-132.

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

25. **Hahn, D., R.I. Amann, W. Ludwig, A.D.L. Akkermans and K.-H. Schleifer**. 1992. Detection of microorganisms in soil after *in situ* hybridization with rRNA-targeted, fluorescently labeled oligonucleotides. Journal of Genetic and Microbiology 138: 879-887.

26. **Hahn, D., M.J.C. Starrenburg and A.D.L. Akkermans**. 1990. Growth increment of *Alnus glutinosa* upon dual inoculation with effective and ineffective *Frankia* strains. Plant and Soil 122: 121-127.

27. **Heller, R.** 1953. Recherches sur la nutrition minérale des tissues végétaux cultivés in vitro, Paris, France.

28. **Hahn, D., M.J.C. Starrenburg and A.D.L. Akkermans**. 1988. Variable compatibility of cloned *Alnus glutinosa* ecotypes against ineffective *Frankia* strains. Plant and Soil 107: 233-243.

29. **Hess, A., P. Höhener, D. Hunkeler and J. Zeyer**. 1996. Bioremediation of a diesel fuel contaminated aquifer: Simulation studies in laboratory aquifer columns. Journal of Contaminated Hydrologie 23: 329-345.

30. **Witty, J.F. and K.E. Giller**. 1991. Evaluation of errors in the measurement of biological nitrogen fixation using ^{15}N fertilizer. In: Stable isotopes in plant nutrition, soil fertility and environmental studies. Vienna: International Atomic Energy Agency.

31. **Hahn, D., R.I. Amann and J. Zeyer**. 1993. Whole-cell hybridization of *Frankia* strains with fluorescence- or digoxigenin-labeled, 16S rRNA-targeted oligonucleotide probes. Applied and Environmental Microbiology 59(6): 1709-1716.

32. **Zarda, B., D. Hahn, A. Chatzinotas, W. Schönhuber, A. Neef, R.I. Amann and J. Zeyer**. 1997. Analysis of bacterial community structure in bulk soil by *in situ* hybridization. Archives of Microbiology 168: 185-192.

33. **Amann, R.I., B.J. Binder, R.J. Olsen, S.W. Chisholm, R. Devereux and D.A. Stahl**. 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Applied and Environmental Microbiology 56: 1919-1925.

34. **Hönerlage, W., D. Hahn, K. Zepp, J. Zeyer and P. Normand**. 1994. A hypervariable 23S rRNA region provides a discriminating target for specific characterization of uncultured and cultured *Frankia*. Systematic and Applied Microbiology 17: 433-443.

35. **Manz, W., R. Amann, W. Ludwig, M. Wagner and K.-H. Schleifer**. 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: Problems and solutions. Systematic and Applied Microbiology 15: 593-600.

36. **Kohls, S.J. and D.D. Baker**. 1989. Effects of substrate nitrate concentration on symbiotic nodule formation in actinorhizal plants. Plant and Soil 118: 171-179.

37. **Zitzer, S.F. and J.O. Dawson**. 1992. Soil properties and actinorhizal vegetation influence nodulation of *Alnus glutinosa* and *Elaeagnus angustifolia* by *Frankia*. Plant and Soil 140: 197-204.

38. **Ekblad, A. and K. Huss-Danell**. 1995. Nitrogen fixation by *Alnus incana* and nitrogen transfer from *A. incana* to *Pinus sylvestris* influenced by macronutrients and ectomycorrhiza. New Phytologist 131: 453-459.

39. **Troelstra, S.R., R. Wagenaar and W. Smant**. 1992. Growth of actinorhizal plants as influenced by the form of N with special reference to *Myrica gale* and *Alnus incana*. Journal of Experimental Botany 43(255): 1349-1359.

40. **Kurdali, F., A.M. Domenach and R. Bardin**. 1990. Alder-poplar associations: Determination of plant nitrogen sources by isotope techniques. Biology and Fertility of Soils 9(4): 321-329.

41. **Asay, K.H., D.A. Johnson and A.J. Palazzo**. 1998. Parent-progeny relationships for carbon isotope discrimination and related characters in crested wheatgrass. International Journal of Plant Sciences 159(5): 821-825.

42. **Raeini Sarjaz, M., N.N. Barthakur, N.P. Arnold and P.J.H. Jones**. 1998. Water stress, water use efficiency, carbon isotope discrimination and leaf gas exchange relationships of the bush bean. Journal of Agronomy and Crop Science 180(3): 173-179.

43. **Paschke, M.W., J.O. Dawson and B.M. Condon**. 1994. *Frankia* in prairie, forest, and cultivated soils of central Illinois, USA. Pedobiologia 38: 546-551.

44. **Dawson, J.O., D.G. Kowalski and P.J. Dart**. 1989. Variation with soil depth, topographic position and host species in the capacity of soils from an Australian locale to nodulate *Casuarina* and *Allocasuarina* seedlings. Plant and Soil 118: 1-11.

45. **Ringø, E., E. Clausen, E. Løvaas, M. Van Ghelue and B. Solheim**. 1995. Effects of extracts of *Alnus glutinosa* seeds on growth of *Frankia*

strain Arl3 under static and fermentor culture conditions. Plant and Soil 176: 283-288.

46. **Zimpfer, J.F., C.A. Smyth and J.O. Dawson.** 1997. The capacity of Jamaican mine spoils, agricultural and forest soils to nodulate *Myrica cerifera*, *Leucaena leucocephala* and *Casuarina cunninghamiana*. Physiologia Plantarum 99: 664-672.

47. **Paschke, M.W. and J.O. Dawson.** 1992. *Frankia* abundance in soils beneath *Betula nigra* and other non-actinorhizal woody plants. Acta Oecologica 13(4): 407-415.

48. **Arveby, A.S. and K. Huss-Danell.** 1988. Presence and dispersal of infective *Frankia* in peat and meadow soils in Sweden. Biology and Fertility of Soils 6(1): 38-44.

49. **Hahn, D.** Personal Communication, 1999.

50. **McEwan, N.R., E.M.O. Gould, E. Lumini, J.D. McNeill and C.T. Wheeler.** 1998. Competitivity and persistence of *Frankia* in a managed environment. Nitrogen fixation with non-legumes. In: Proceedings of the 7th international symposium on nitrogen fixation with non-legumes, Eds.: A. Malik, M.S. Mirza, and J.K. Ladha, Kluwer Academic Publishers, Dordrecht: 315-326.

Chapter 5

Saprophytic Growth of *Frankia*

Seite Leer /
Blank leaf

Chapter 5

Saprophytic Growth of *Frankia*

5.1. Abstract

The ability of *Frankia* to grow saprophytically in different soils without host plants was investigated. Soils were inoculated with *Frankia* pure cultures Arl3 (alder host infection group IIIa) and Ag45/Mut15 (alder host infection group IV). Half of each soil was then mixed with ground dried alder leaves and incubated for 14 weeks. Samples were taken every 1-4 weeks and DNA was extracted. Total *Frankia* DNA content as well as DNA content from alder host infection group IIIa and IV was determined by dot blot hybridizations with specific probes. The results showed that (i) *Frankia* is able to grow saprophytically, (ii) the addition of dried alder leaves had some effect on the *Frankia* population in the soil, and that (iii) there are differences between the strains regarding the ability to grow under the applied conditions.

5.2. Introduction

Actinomycetes of the genus *Frankia* are able to live in symbiosis with woody host plants (actinorhizal plants) such as *Alnus* spp. (alders). They induce nodules on the roots of the plants in which nitrogen in fixed. Consequently, the host plants are largely independent of soil nitrogen and able to grow on nitrogen-poor soils. *Alnus* spp. have been used for the production of timber and fuelwood as well as for soil restoration [1] [2] [3]. In addition they are used as nurse trees in interplantings where they enhance the growth of the associated vegetation through nitrogen fertilization [4]. Therefore they are an important component of the ecosystem and economy.

Although *Frankia* can be found in the rhizosphere and in bulk soil most studies to date focussed on the biology of *Frankia* in root nodules. Consequently, little is known regarding the biology of *Frankia* in soil. *Frankia* spp. occur widely in soil lacking host plants [5] [6] [7] [8] [9]. They are surprisingly abundant beneath

certain non-host species such as birch [10] [11], demonstrating that *Frankia* populations can survive and remain infective in soils that are devoid of host plants. These observations as well as the fact that *Frankia* can be grown in pure cultures indirectly suggest that *Frankia* can grow saprophytically in soil. However, the means by which *Frankia* survive in the soil are poorly understood and there are no data available that would prove that *Frankia* is able to grow saprophytically in the soil. *Frankia* may not grow but merely persist in the soil in a non-growing but viable form such as spores.

Investigations on the ecology of *Frankia* populations in soil are usually done by plant bioassays in which a host plant is used to trap the infective *Frankia* population in the soil [12] [13] or in serial dilutions of the soil [14]. Valuable information about the nodulation capacity of a soil is provided by this method. However, as the number of potential infection sites on root systems limits nodulation [15], the number of infective *Frankia* units may be underestimated especially when the number of infective *Frankia* is high. A further drawback of this method is its selectivity. Only nodule-forming *Frankia* populations are detected and the physiologically inactive fraction of *Frankia* in the soil, which does not form nodules [5], is neglected by this approach. In addition, soil characteristics are known to affect nodulation. The most commonly reported influential characteristics are nitrate concentration [16], pH [17], and soil moisture [12] [18]. Since only one successful isolation from soil has been reported [19], molecular methods which are independent of isolation and cultivation have to be used for studies on he biology of *Frankia* in soil.

It could be shown that two introduced *Frankia* strains (Arl3 and Ag45/Mut15) can not only survive in a soil without host plants but also compete for nodule formation with the indigenous population when alder seedlings are planted. The addition of dried alder leaves to the soil resulted in a higher N_2-fixing activity as well as in a totally changed *Frankia* population in the nodules [20]. Consequently, it can be assumed that the treatment with alder leaves promoted saprophytic growth of a fraction of the *Frankia* soil population.

For future planting of *Alnus* spp. for the purpose of nitrogen fertilization and soil restoration it would be valuable to know whether (i) *Frankia* can grow saprophytically in a soil, and (ii) the growth of a *Frankia* population can be

promoted by added substrate. It was the aim of this study to analyze the fate of introduced *Frankia* strains in soils devoid of host plants and to determine the effect of the addition of organic material (tdried alder leaves) to the soil on the *Frankia* population using molecular methods.

5.3. Material and Methods

5.3.1. Characteristics of soil samples

Surface samples (to a depth of 20 cm) from the silty clay *Hau* (Birmensdorf, Switzerland) (high content of organic material) [21] and the sandy loam *Ettiswil* (Ettiswil, Switzerland) (low content of organic material) [20, 22, 23] were collected in September 1998 (Table 5.1). Freshly sampled soil was cleared of larger particles, e.g. roots and stones, sieved (mesh size 5 mm), and stored at 4 °C for 6 weeks.

Table 5.1. Soil characteristics

Soil	Texture %clay/silt/sand	Org. material %	Dry weight %	pH (in water)
Ettiswil	nd[a]	0.02	76.4	7.1
Hau	41/52/7	5.6	68.8	6.8

[a] not determined

Soil *Ettiswil* contains an indigenous *Frankia* population consisting of *Alnus* host infection groups IIIa, IIIb and IV [22, 24]. *Frankia* populations in soil *Hau* were not determined.

5.3.2. Cultivation and preparation of *Frankia* strains

Frankia strains Arl3 and Ag45/Mut15 were grown for 4 weeks in P+N-medium [25] containing sodium propionate and ammonium chloride as C- and N-source, respectively. Cultures were harvested by centrifugation, washed twice in

phosphate buffered saline (PBS, composed of 0.13 M NaCl, 7 mM Na_2HPO_4 and 3 mM NaH_2PO_4, pH 7.2 in water) [26] and homogenized in PBS by repeated passages through a needle (0.6 mm in diameter) with a sterile syringe [27].

5.3.3. Experimental Set-Up

Soils were inoculated with a mixture of pure cultures of *Frankia* strains Arl3 and Ag45/Mut15 each in an estimated density of 10^7 cells g^{-1} soil (fresh weight). Half of each inoculated soil was mixed with ground dried alder leaves. Leaves were collected directly from alders at a natural alder stand not identical with the sites on which the soils were harvested and dried at 120 °C for 3 days. The other half of each soil without additional leaves was used as control. The soils were incubated in a climate chamber for 14 weeks (16 h day/8 h night, 20 °C/16 °C). Soil samples were harvested before inoculation, directly after inoculation (time = 0) and after 1, 2, 3, 4, 6, 10, and 14 weeks of incubation. Water content in the incubated soils was kept in the range of 26-33% and 32-37% for soils *Ettiswil* and *Hau*, respectively, by weighting the pots and adding water as necessary.

5.3.4. Analysis of soil parameters

Anion concentrations (NO_3^- and NO_2^-) from pore water of soil samples were determined by ion chromatography (Dionex DX-100 ion chromatograph equipped with an IonPac AS4A-SC column; Dionex, Sunnyvale, USA) [28]. The pore water was obtained by centrifugation of approximately 12 g of soil filled into 5 ml plastic syringes which were plugged with silane treated glass wool (Supelco Inc., Bellefonte, USA). The filled syringes were placed in 15 ml Falcon tubes and centrifuged (2500 x g) for 10 min at 4°C. Anion concentrations in pore water (µM) were correlated to water contents and expressed in µmol g $soil^{-1}$ (dry weight).

5.3.5. DNA extraction procedure

Nucleic acid extraction from soil samples was based on a modified beat beating protocol [29]. The homogenization mixture contained 0.4 g soil (wet weight), 0.75 g glass beads (0.1 – 0.11 mm, Braun, Melsungen, Germany) and 1.6 ml of a modified extraction buffer previously described for RNA extraction (0.2% hexadecyltrimethylammonium bromide (CTAB), 1mM dithiotreitol (DTT), 0.2 M sodium phosphate buffer (pH 8), 0.1 M NaCl and 50 mM EDTA) [30] in a 2 ml Eppendorf tube. Cells were disrupted in a bead beater (Fastprep FP120 bead-beater, Bio101, Savant, Vista CA, Farmingdale, NY) in a 45-second-burst at a beating speed of 5.0 ms^{-1}, corresponding to approx. 5250 rpm according to the manufacturer. Released nucleic acids in the soil homogenates were further purified after centrifugation in an Eppendorf centrifuge at 14000 rpm for 5 min. The supernatant was extracted with an equal amount of phenol/chloroform (1/1), followed by precipitation of the nucleic acids with 20% Polyethylenglycol 6000 and 2.5 M NaCl (1h, 37 °C) [31] [32]. DNA was pelleted by centrifugation at 14000 rpm for 30 min. Pellets were washed once with 70% ethanol and resuspended in 300µl TE (10 mM TRIS-HCl, 1 mM EDTA, pH 8). After subsequent treatment with RNase A (Fluka, Buchs, Switzerland; 95.5 U mg^{-1}, final concentration 0.1 µg $µl^{-1}$) at 37 °C for 30 min, the DNA was quantified with PicoGreen (see below). DNA was further purified by centrifugation through Sephadex G-200 columns (1 ml syringes filled with Sephadex G-200 to 1 ml) [33], again extracted with phenol/chloroform, precipitated, dried and resuspended in 100µl TE (10 mM TRIS-HCl, 1 mM EDTA, pH 8). DNA of extracts was visualized by gel electrophoresis and the total amount of purified DNA was quantified.

5.3.6. Quantification of DNA

DNA in the soil extracts was quantified with PicoGreen [34]. Briefly, 1 µl of DNA extract, 2 µl of PicoGreen (Molecular Probes, Eugene, OR) and 397 µl of TE were mixed in a polypropylene tube, vortexed and fluorescence was quantified in a luminescence spectrometer (Perkin Elmer LS 50 B, U.K.). Standards were prepared from bacteriophage lambda DNA stocks (Appligene, Basel,

Switzerland). DNA yield was calculated as µg total DNA in solution g^{-1} extracted soil (dry weight).

5.3.7. Dot blot hybridization

For dot blot hybridization, nucleic acid samples corresponding to the DNA content of 0.1 g dry soil were denatured with NaOH (final concentration 0.2 M) for 20 min at room temperature. After neutralization with ammonium acetate (final concentration 0.2 M), nucleic acids (final volume 60 – 400 µl) were applied to nylon filters (Boehringer, Mannheim, Germany) with a dot blot manifold (BioRad, Hercules, CA) and eventually bound to the membrane at 120 °C for 30 min. Membranes were hybridized overnight with digoxigenin-labeled probes [24] (Table 5.2) in standard buffer (5 x SSC, 0.1% N-lauroyl-sarcosine, 0.02% SDS, 1% blocking reagent).

Table 5.2. Probes used for dot blot hybridization

Probe	Specificity	Strain	Sequence (5'->3')	Hybridization temperature
23Fra	Genus *Frankia*	All *Frankia*	ATCGCATGCCTACTACC	50 °C
23Arl3	*Frankia* of the *Alnus* host infection group IIIa[a]	Arl3	CCAGACACATCTCCGAAAAG	58 °C
23Mut(II)	*Frankia* of the *Alnus* host infection group IV[a]	Ag45/Mut15	CCACACACACCCCCTAA	63 °C

[a] According to the classification in [35]

The formation of stable hybrids was shown by binding of an antibody-alkaline phosphatase conjugate (Boehringer, Mannheim, Germany) to the digoxigenin reporter molecule. Alkaline phosphatase activity was visualized by light emission using CSPD-Star™ chemoluminescent substrate (Boehringer,

Mannheim, Germany) and exposure to Kodak X-OMAT AR film according to the manufacturers instructions (Boehringer, Mannheim, Germany).

5.3.8. Quantification of signal intensities

Signal intensities were determined by image analysis of films in a gel documentation system (GelDoc 2000, BioRad).

Since no serial dilution of pure culture DNA was hybridized no calibration curve could be determined. Consequently, a correlation between cell numbers of pure cultures and DNA extracted from soil is not possible. Therefore, only relative amounts of DNA were calculated. Intensities obtained with group specific probes 23Arl3 and 23Mut(II) on DNA of pure cultures were set 100%. Signal intensities from DNA extracted from soil samples were compared to those signals obtained from pure culture DNA. Relative amounts of DNA from *Alnus* host infection group IIIa and IV from the extracted soils were calculated by comparing the intensity of the soil DNA signal to that of the pure cultures Arl3 and Ag45/Mut15, respectively, each hybridized to its specific probe. Relative amounts of total *Frankia* DNA from the extracted soils were calculated by comparing the intensity of the soil DNA signal to that of the pure culture Ag45/Mut15 hybridized to probe 23Fra. It was assumed that the signal intensity of probe 23Fra hybridized to DNA obtained from a defined number of cells of strain Ag45/Mut15 is similar to the signal intensity of probe 23Fra hybridized to DNA extracted from the same number of mixed *Frankia* cells.

5.4. Results and Discussion

5.4.1. Soil parameters

Water content increased slightly in all soils during the 14 weeks of incubation from 26% to 33% in soil *Ettiswil* and from 32% to 37% in soil *Hau*. The nitrite content of the soil exhibited a parallel increase from 0-250 µmol g^{-1} soil (dry weight) and 0-240 µmol g^{-1} soil (dry weight) in soils *Hau* and *Ettiswil*, respectively. Nitrate content in the soils decreased (Fig. 5.1), indicating microbial denitrification activity. In soil *Hau* as well as in soil *Ettiswil*, the

addition of dried alder leaves to the soil resulted in a temporal decrease in the nitrate content compared to soils without leaves (Fig. 5.1). This may have been caused by the degradation of the added organic material by the microbial community using NO_3^- as oxidant and subsequent release of the NO_3^- when cells died. Therefore saprophytic growth of *Frankia* might not only be influenced by changed concentrations of organic material or chemical substances in the leaves but also by different nitrate concentrations in the two treatments.

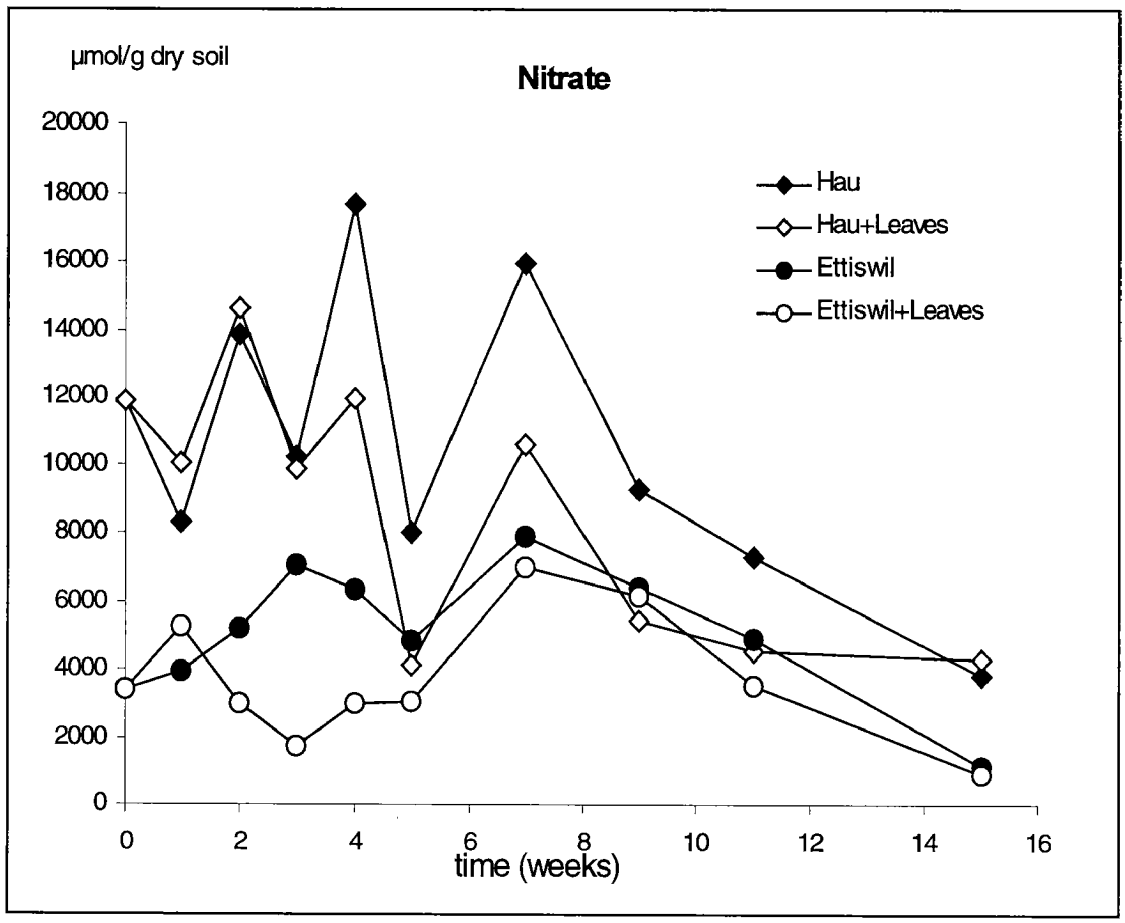


Figure 5.1. Nitrate concentration in soils during the experiment. No error bars are given since no replicates were taken.

5.4.2. DNA extraction

A necessary prerequisite for filter hybridization is the extraction and purification of DNA from the soil. The total amount of DNA extracted from samples taken during incubation time was between 17 and 78 µg for soil *Hau*, and between 7 and 34 µg for soil *Ettiswil* (after purification). Although several protocols for

DNA extraction from soil exist [29] [36] [37], a major drawback of all methods is that the extracts may not represent the living bacterial population in the soil. The possible isolation of free nucleic acids from dead cells or DNA sorbed to soil minerals must be taken into account. In addition, quantitative recovery of nucleic acids from soil may be hampered by some types of microbial cells such as spores which could resist lysis. However, intensive methods, such as the bead-beating procedure used in this study, can be assumed to be effective, although some degree of DNA shearing cannot be avoided [38]. Purification, which is necessary to remove hybridization inhibiting substances, leads to DNA losses although the use of Sephadex columns results in relatively high levels of DNA recovery [39].

5.4.3. Dot blot hybridizations

The hybridizations with the probe specific for *Frankia* (23Fra) resulted in positive signals for all tested *Frankia* pure cultures (Arl3, and Ag45/Mut15), while a pure culture of the closely related *Streptomyces azureus* gave no signal. Probes specific for the *Alnus* host infection group IIIa (23Arl3) and IV (23Mut(II) resulted in positive signals for the corresponding reference pure cultures and negative signals for the other *Frankia* pure culture tested (Fig. 5.2).

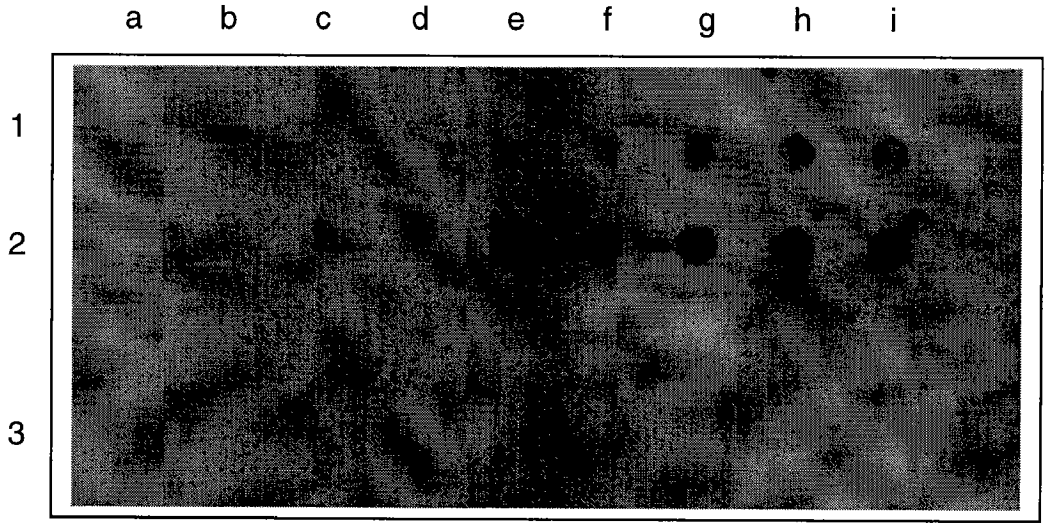


Figure 5.2. Hybridization with probe 23Mut(II) on DNA extracted from soil *Ettiswil*. 1a: non-inoculated soil; 1b-i: Soil at weeks 0, 1, 2, 3, 4, 6, 10, 14; 2b-i: Soil with leaves at weeks 0, 1, 2, 3, 4, 6, 10, 14; 3a: *S. azureus*; 3b: Arl3; 3c: Ag45/Mut15; 3h: Ag45/Mut15+non-inoculated soil.

5.4.4. Soil *Hau*

Probe 23Fra

With the *Frankia* specific probe 23Fra no trend could be observed except a slight signal decrease in the last 10 weeks of the experiment (Fig. 5.3.a). In the first 4 weeks, the *Frankia* DNA content in the soil fluctuated. This could possibly be explained by the growth of *Frankia* in hyphae which are difficult to disrupt completely. The resulting unequal distribution of *Frankia* cells in the soil may result in hot spots with high *Frankia* density and consequently high *Frankia* DNA amounts, while other sites harbor only a few or no *Frankia* cells. This problem could be avoided by sampling in replicates, which would not only allow a calculation of standard deviations but also significant differences. There was no difference in hybridization signal intensities between soil with alder leaves and soil without alder leaves.

Probe 23Arl3

Using a probe specific for *Alnus* host infection group IIIa (23Arl3), a difference between the two treatments could be detected in the first 4 weeks of the experiment. The addition of leaves resulted in a decrease in *Frankia* DNA from this group in the first weeks. Afterwards the DNA content increased again to the initial values. DNA concentration in soil without leaves fluctuated slightly, but again the concentration at the end of the experiment are comparable to those at the beginning (Fig. 5.3.b).

Probe 23Mut(II)

Using a probe specific for *Alnus* host infection group IV a slight increase in signal intensity over time could be observed. Obviously, the DNA concentration of *Frankia* strains belonging to this group increased during the incubation, as the DNA concentration at the end of the experiment was about twice the beginning concentration (Fig. 5.3.c). There were only minor differences between the treatments.

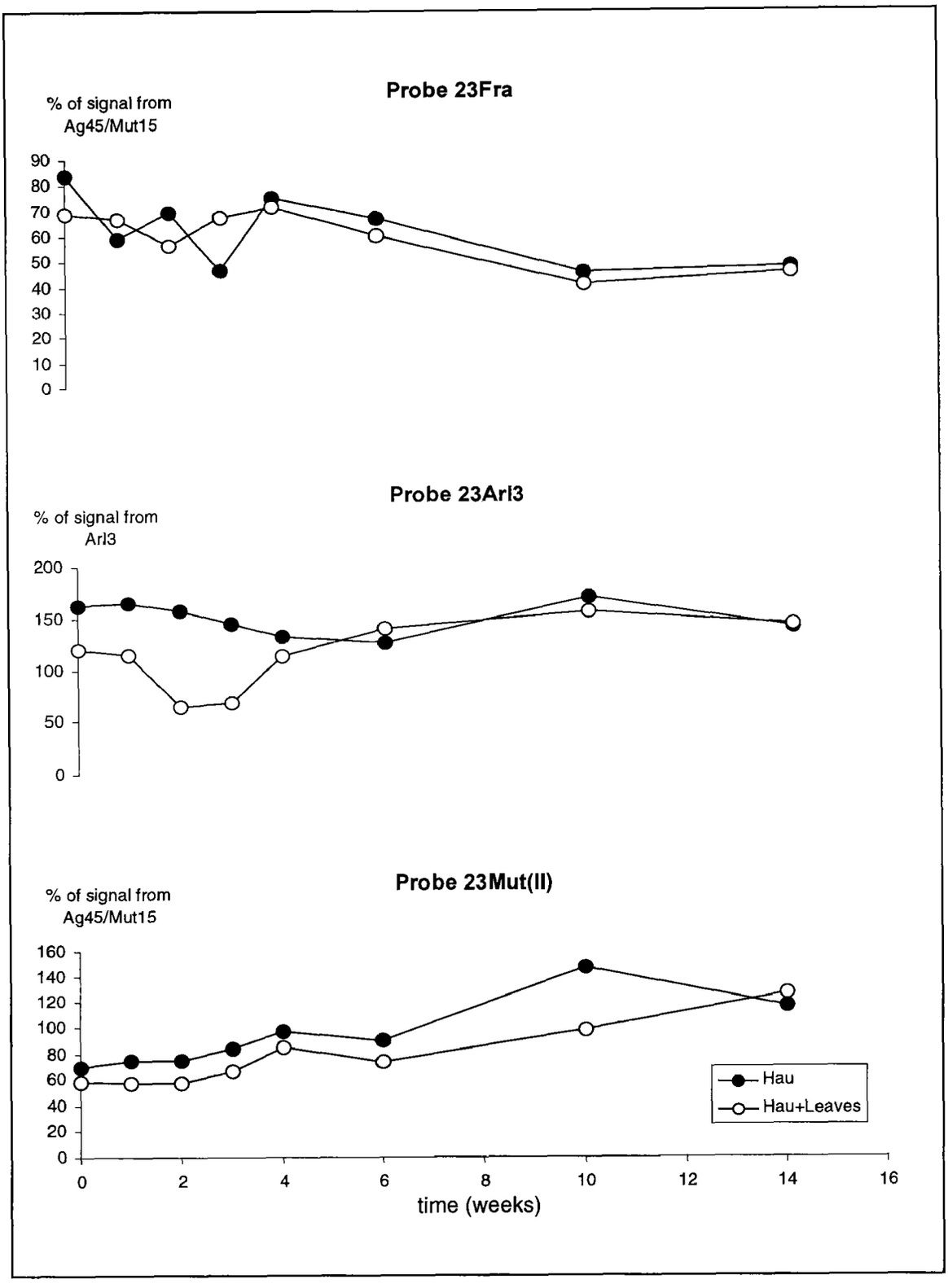


Figure 5.3. Hybridizations of DNA extracted from soil *Hau* with probes 23Fra, 23Arl3 and 23Mut(II). Since no replicates were sampled, no error bars can be given.

5.4.5. Soil *Ettiswil*

Probe 23Fra

Using the *Frankia* specific probe 23Fra an increase in *Frankia* DNA could be detected (Fig. 5.4.a). Compared to the *Frankia* DNA content at the beginning of the experiment a 2.5-3-fold measure in DNA could be detected after 14 weeks of incubation. No differences between the soil with leaves and the soil without leaves could be observed.

Probe 23Arl3

Hybridizations with probe 23Arl3 showed a slight increase in DNA of *Frankia* belonging to *Alnus* host infection group IIIa (Fig. 5.4.b). Differences between the treatments were small, the addition of leaves to the soil possibly promoted the growth of a strain belonging to this *Alnus* host infection group.

Probe 23Mut(II)

Using probe 23Mut(II) a five-fold increase in DNA of *Frankia* belonging to *Alnus* host infection group IV could be detected in soil with and without leaves (Fig. 5.4.c). Here the *Frankia* growth promotion in the first six weeks of the experiment by the addition of dried alder leaves was obvious compared to the soil without leaves. However, towards the end of the experiment DNA-concentrations of *Frankia* belonging to this *Alnus* host infection group was similar in both treatments.

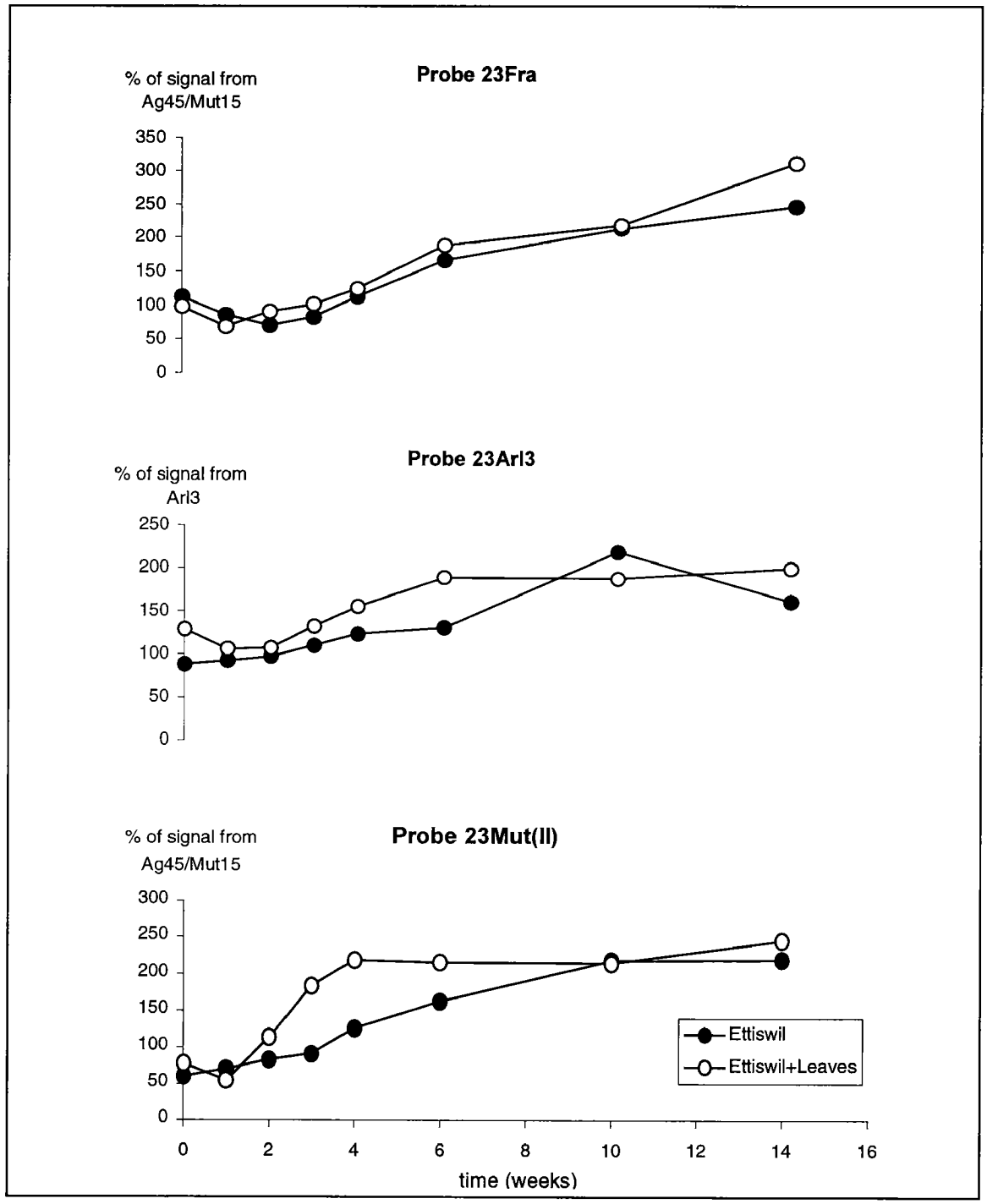


Figure 5.4. Hybridizations of DNA extracted from soil *Ettiswil* with probes 23Fra, 23Arl3 and 23Mut(II). Since no replicates were sampled, no error bars can be given.

The increase in DNA from *Alnus* host infection group IV in both soils indicates that a strain belonging to this group can grow saprophytically under the applied conditions. Addition of dried alder leaves to soil *Ettiswil* under the same storage conditions resulted in higher percentages of a strain belonging to *Alnus* host infection group IV in nodules [20]. There are several possible reasons for this effect. The lower nitrate concentration in soils with leaves in the first weeks of incubation could have an effect on the competitive abilities of this strain or decrease the competitive fitness of other strains. Another possible explanation would be the higher amount of organic material in soils with leaves, since it has been observed that concentration of organic matter in the soil is positively correlated with the density of *Frankia* [40]. The increased growth rate in soils with leaves compared to soils without leaves could also be caused by some substances present in the alder leaves. It has been demonstrated that green *Casuarina* cladodes have a positive effect on the growth of *Casuarina* infective *Frankia* in soil and pure cultures [9]. Another possible reason for the better competitivity of strain Ag45/Mut15 compared to strain Arl3 might be that Ag45/Mut15 grows faster than Arl3 at room temperature [41], since the soil was stored between 16°C and 20°C.

5.5. Conclusion

We do have some evidence for our original hypothesis, that *Frankia* is able to grow saprophytically in soils without host plants. However, to give reliable statements the preparation of hybridization replicates would have been necessary. This would enable the calculation of standard deviations and therefore significant differences. Nevertheless, qualitative conclusions can be made. The increase in DNA from group IV in both soils, *Hau* and *Ettiswil*, indicates that a strain belonging to this group can grow saprophytically under the applied conditions. In contrast a strain belonging to *Alnus* host infection group IIIa was not able to grow as well under the parameters tested. The question whether this different growth rates were caused by a higher content of organic material or a lower nitrate concentration in soils with leaves, or substances present in the leaves remains open.

5.6. References

1. **Goldman, C.R.** 1961. The contribution of Alder trees (*Alnus tenuifolia*) to the primary productivity of Castle lake, California. Ecology 42(2): 282-288.

2. **Huss-Danell, K. and H. Ohlsson**. 1992. Distribution of biomass and nitrogen among plant parts and soil nitrogen in a young *Alnus incana* stand. Canadian Journal of Botany 70: 1545-1549.

3. **Mikola, P.** 1975. Afforestation of bogs after industrial exploitation of peat. Silva Fenn 9: 101-115.

4. **Dawson, J.O. and J.W. Van Sambeck**. 1993. Interplanting woody nurse crops promotes differential growth of black walnut saplings. In: Proceedings, 9th Central Hardwood Forest Conference, Eds.: A.R. Gillespie, P.E. Pope, G. Rink, U.S. Department of Agriculture, Forest Service, North Central Forest Experiment Station, West Lafayette: 455-463.

5. **Maunuksela, L., K. Zepp, T. Koivula, J. Zeyer, K. Haahtela and D. Hahn**. 1999. Analysis of *Frankia* in three soils devoid of actinorhizal plants. FEMS Microbiology Ecology 28: 11-21.

6. **Paschke, M.W. and J.O. Dawson**. 1992. The occurrence of *Frankia* in tropical forest soils of Costa Rica. Plant and Soil 142: 63-67.

7. **Smolander, A. and V. Sundman**. 1987. *Frankia* in acid soils of forests devoid of actinorhizal plants. Physiologia Plantarum 70: 297-303.

8. **Smolander, A.** 1990. *Frankia* populations in soils under different tree species - with special emphasis on soil under *Betula pendula*. Plant and Soil 121: 1-10.

9. **Zimpfer, J.F., G.J. Kennedy, C.A. Smyth, J. Hamelin, E. Navarro and J.O. Dawson**. 1998. Localization of *Casuarina*-infective *Frankia* near *Casuarina cunninghamiana* trees in Jamaica. Canadian Journal of Botany, 77(9): 1248-1256.

10. **Paschke, M.W. and J.O. Dawson**. 1992. *Frankia* abundance in soils beneath *Betula nigra* and other non-actinorhizal woody plants. Acta Oecologica 13(4): 407-415.

11. **Smolander, A. and M.L. Sarsa**. 1990. *Frankia* strains of soils under *Betula pendula*: Behaviour in soil and pure culture. Plant and Soil 122: 129-136.

12. **Dawson, J.O., D.G. Kowalski and P.J. Dart**. 1989. Variation with soil depth, topographic position and host species in the capacity of soils from an Australian locale to nodulate *Casuarina* and *Allocasuarina* seedlings. Plant and Soil 118: 1-11.

13. **Kurdali, F. and A.M. Domenach**. 1991. Evaluation of nodulation capacity in *Alnus glutinosa* seedlings grown in soil sampled from various depths. Acta Oecologica 12: 397-401.

14. **Paschke, M.W., J.O. Dawson and B.M. Condon**. 1994. *Frankia* in prairie, forest, and cultivated soils of central Illinois, USA. Pedobiologia 38: 546-551.

15. **Quispel, A.** 1954. Symbiotic nitrogen fixation in non-leguminous plants - the influence of the inoculation density and external factors on the nodulation of *Alnus glutinosa* and its importance to our understanding of the mechanism of the infection. Acta Botanica Neerlandica 3(4): 512-532.

16. **Kohls, S.J. and D.D. Baker**. 1989. Effects of substrate nitrate concentration on symbiotic nodule formation in actinorhizal plants. Plant and Soil 118: 171-179.

17. **Igual, J.M., C. Rodriguez-Barrueco and E. Cervantes**. 1997. The effects of aluminum on nodulation and symbiotic nitrogen fixation in *Casuarina cunninghamiana* Miq. Plant and Soil 190: 41-46.

18. **Pratt, S.D., A.S. Konopka, M.A. Murry, F.W. Ewers and S.D. Davis**. 1997. Influence of soil moisture on the nodulation of post fire seedlings of *Ceanothus* spp. growing in the Santa Monica Mountains of Southern California. Physiologia Plantarum 99: 673-679.

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

20. **Nickel, A., O. Pelz, D. Hahn, M. Saurer, R. Siegwolf and J. Zeyer**. In preparation. Use of molecular and isotope techniques to study the *Frankia*-Alder symbiosis under different environmental conditions.

21. **Richard, F., P. Lüscher and T. Strobel**. 1978. Physikalische Eigenschaften von Böden der Schweiz, Eds.: Richard, F., P. Lüscher and T. Strobel, Eidgenössische Anstalt für das forstliche Versuchswesen: Birmensdorf, Switzerland.

22. **Zepp, K., D. Hahn and J. Zeyer**. 1997. *In situ* analysis of introduced and indigenous *Frankia* populations in soil and root nodules obtained on *Alnus glutinosa*. Soil Biology and Biochemistry 29(9/10): 1595-1600.

23. **Nickel, A., D. Hahn, K. Zepp and J. Zeyer**. 1999. *In situ* analysis of introduced *Frankia* populations in root nodules of *Alnus glutinosa* grown under different water availability. Canadian Journal of Botany 77(9): 1231-1238.

24. **Zepp, K., D. Hahn and J. Zeyer**. 1997. Evaluation of a 23S rRNA insertion as target for the analysis of uncultured *Frankia* populations in root nodules of alders by whole cell hybridization. Systematic and Applied Microbiology 20: 124-132.

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

26. **Hahn, D., R.I. Amann, W. Ludwig, A.D.L. Akkermans and K.-H. Schleifer**. 1992. Detection of microorganisms in soil after *in situ* hybridization with rRNA-targeted, fluorescently labeled oligonucleotides. Journal of Genetic and Microbiology 138: 879-887.

27. **Hahn, D., M.J.C. Starrenburg and A.D.L. Akkermans**. 1990. Growth increment of *Alnus glutinosa* upon dual inoculation with effective and ineffective *Frankia* strains. Plant and Soil 122: 121-127.

28. **Hess, A., P. Höhener, D. Hunkeler and J. Zeyer**. 1996. Bioremediation of a diesel fuel contaminated aquifer: Simulation studies in laboratory aquifer columns. Journal of Contaminated Hydrologie 23: 329-345.

29. **Bürgmann, H., M. Pesaro, F. Widmer and J. Zeyer**. In preparation. Optimizing bead beating procedures for DNA extraction from soil.

30. **Cheung, A.L., K.J. Egerhardt and V.A. Fischetti**. 1994. A method to isolate RNA from gram-positive bacteria and mycobacteria. Anal. Biochem. 222(2): 511-514.

31. **Rowland, L.J. and B. Nguyen**. 1993. Use of polyethylene-glycol for ourification of DNA from leaf tissue of woody plants. Biotechniques 14(5): 734.

32. **Yeates, C., M.R. Gillings, A.D. Davison, N. Altavilla and D.A. Veal**. 1997. PCR amplification of crude microbial DNA extracted from soil. Letters in Applied Microbiology 25(4): 303-307.

33. **Tsai, Y.-L. and B. Olsen**. 1992. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Applied and Environmental Microbiology 58: 2292-2295.

34. **Sandaa, R.A., O. Enger and V. Torsvik**. 1998. Rapid method for fluorometric quantification of DNA in soil. Soil Biology and Biochemistry 30(2): 265-268.

35. **Hönerlage, W., D. Hahn, K. Zepp, J. Zeyer and P. Normand**. 1994. A hypervariable 23S rRNA region provides a discriminating target for specific characterization of uncultured and cultured *Frankia*. Systematic and Applied Microbiology 17: 433-443.

36. **Purdy, K.J., T.M. Embley, S. Takii and D.B. Nedwell**. 1996. Rapid extraction of DNA and rRNA from sediments by a novel hydroxyapatite spin-column method. Applied and Environmental Microbiology 62(10): 3905-3907.

37. **Smalla, K., N. Cresswell, L.C. Mendoca-Hagler, A. Wolters and J.D. Van Helsas**. 1993. Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. Journal of Applied Bacteriology 74: 78-85.

38. **Wintzingerode, F., U.B. Göbel and E. Stackebrandt**. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiology Reviews 21: 213-229.

39. **Miller, D.N., J.E. Bryant, E.L. Madsen and W.C. Ghiorse**. 1999. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Applied and Environmental Microbiology 65(11): 4715-4724.

40. **Nalin, R., P. Normand and A.M. Domenach**. 1997. Distribution and N_2-fixing activity of *Frankia*-strains in relation to soil depth. Physiologia Plantarum 99: 732-738.

41. **Hahn, D.** Personal Communication, 1999.

Seite Leer /
Blank leaf

Chapter 6

Discussion

Seite Leer /
Blank leaf

Chapter 6

Discussion

6.1. Greenhouse versus field experiments

An improvement of the symbiosis with the result of a higher N_2-fixation rate is economically interesting [1]. This can only be achieved by a better understanding of the processes in soil and nodules.

Experiments on nodulation capacity and N_2-fixation have been carried out in different systems with variable degrees of complexity. Plants have been grown in nutrient solution with defined composition [2] [3] [4], in sterile substrates [5] [6], in natural soils transferred to pots [7] [8] [9] or in the field [10] [11]. All experiments presented in this thesis were carried out using natural soil in pots since the results were intended to be applicable to the field.

Pot experiments seem to be a satisfactory compromise between axenic cultures in defined nutrient solutions and the complex situation in the field. Greenhouse studies using natural soil with an indigenous microbial population are more complex than sterile nutrient solutions, resulting in a better representation of the natural field conditions. In addition, it is only possible in such defined systems to control environmental factors such as light, temperature and water content of the soil and to keep these parameters constant. This reduces the number of factors influencing nodulation and N_2-fixation [12] [13] and consequently simplifies relating the results to field situations. On one hand, the various factors in the field do have an influence on the symbiosis, on the other hand they can not be predicted or measured [14]. These factors make greenhouse studies essential to understand the situation in the field.

Although such greenhouse experiments are essential to demonstrate the possible effects measured in the field, they also have disadvantages. The primary drawback of pot experiments is that the space in the greenhouse as well as in the pots in which plants are growing is restricted so that plants must

be harvested after a certain time. This does not allow conclusions about the long-term development of either plant growth [15] [16] or *Frankia* populations in the soil. In addition, it is difficult to relate results obtained in restricted systems to the field and to predict the field performance of a *Frankia*-host tree symbiosis. This problem, however, cannot neither be solved by making field experiments since relating results observed at one field site to another field site, where soil, microbial population and other environmental factors might be completely different, is difficult [17] [15].

6.2. Molecular Methods

Molecular techniques that rely on DNA hybridization-based reactions such as PCR, dot blot hybridization or whole cell hybridization can be used to detect and identify microorganisms in their natural habitat without prior cultivation. Hence, they are suitable to be used in ecological studies [18]. They have been applied previously in *Frankia* research and today, various general and specific probes and primers for the identification of frankiae are available [19] [20] [21] [22] [23] [24] [25].

However, instead of being strain-specific, most of the probes available for whole cell hybridization are either group-specific, specific for all strains which are able to fix molecular nitrogen [21], specific depending on the host plant [24], or specific for *Alnus* host infection groups [23]. Consequently, e.g. in the case of the *Alnus* infective strains used in the experiments in this thesis, the probes can only be used to identify groups and not single strains. This makes it impossible to distinguish between an introduced strain and an indigenous strain that belong to the same host infection group. This restriction would be necessary to follow the fate of the introduced strain and to study its ability to compete with the indigenous population and to persist in the soil. The problem can be partly solved by growing parallel control plants on non-inoculated soil containing only the natural *Frankia* population and by comparing the inoculated and the non-inoculated treatment.

6.3. Stable isotope measurements (^{15}N)

The use of the heavy isotope ^{15}N is a very useful tool to determine the activity of N_2-fixing microorganisms. The heavy isotope can either be added as $^{15}N_2$, or in the form of ^{15}N-labeled nitrate or ammonium to the soil or water (isotope dilution method) [26].

In this study, the N_2-fixing activity was determined by the stable isotope dilution method, where ^{15}N-labeled nitrate is added to the soil (Chapter 4). This method requires a non-N_2-fixing reference plant and relies on two major assumptions: (i) that both, test plant and reference plant, take up unlabeled N from the soil and labeled N from the fertilizer in proportion to the available amounts, and (ii) that the ratio of labeled fertilizer N to unlabeled soil N available to the two plants is the same [26]. Consequently, if one or both of those assumptions are not fulfilled, inaccurate estimates of N_2-fixation rates will be obtained. This is the case when there are species-specific differences between test plant and reference plant regarding N uptake. Such a situation is given when e.g. N_2-fixation of alders is to be determined and the reference plant is no alder. This is also possible when there are temporal differences in N uptake between test plant and reference plant that are related to the stage of plant development. This could have happened in the experiment in this study where nodulated and non-nodulated alder were used [27]. As a result of the mineralization process there is a gradual decline in enrichment of the soil-plus-fertilizer N pool, thus differences in timing of uptake of N from this pool are important [28]. In addition, soil N uptake is different in N_2-fixing *Casuarina* than in non-nodulated *Casuarina* [29].

Therefore the choice of the reference plant is extremely important [28]. In fact, the values for N_2-fixation of alders obtained with the isotope dilution technique are reported to be variable. They depend not only on the individual alder seedling but also on the reference plant. There are considerable differences in the rate of N_2-fixation when non-nodulated alder is used as reference plant when compared to calculated rates when a grass (*Festuca rubra*, red fescue) is used as reference plant [27]. Another important point is that the incubation

period must be long enough to observe sufficient ^{15}N uptake since small ^{15}N uptake results in very large errors [30] [31].

Provided that these drawbacks are taken into account the ^{15}N isotope dilution method is a reliable method to select the most effective N_2-fixing *Frankia*–plant combinations and the best environmental conditions [32] [33].

6.4. Effect of inoculation on plant growth, N_2-fixation and *Frankia* population

The inoculation with *Frankia* pure cultures promoted plant growth in all performed experiments. This is consistent with other studies [6] [34] [35]. Plant growth was positively correlated with increased N_2-fixation, which was indicated in the first experiment (Chapter 3) by the formation of small, non-functional nodules on non-inoculated trees. In the second experiment (Chapter 4) the increased N_2-fixation could be directly determined the by the use of the stable ^{15}N isotope.

The efficiency of the symbiosis was increased after inoculation although the soil used in these studies contained an active indigenous *Frankia* population. However, it has been shown that only a small fraction of the *Frankia* population in the soil is able to form nodules [36] [9]. The reason for this is probably that only the physiologically active *Frankia* in the soil can induce nodules [37]. Consequently, when introduced at the time of planting (Chapter 3), the physiologically active pure cultures had a competitive advantage over the indigenous population, which might have been largely inactive. To overcome this problem, another experiment was carried out, in which pure cultures were introduced into the soil several weeks before *Alnus glutinosa* seedlings were planted (Chapter 4). In this experiment the pure cultures had to not only adapt to the conditions in the soil but also compete with the indigenous population. Again, inoculation significantly promoted *A. glutinosa* growth, although this time the difference between the inoculated and the non-inoculated treatment was smaller (Chapter 3 and 4). In both cases, inoculation before and at the time of planting, addition of pure cultures resulted in a changed *Frankia* population in

the nodules compared to non-inoculated counterparts. While nodules from non-inoculated trees contained only *Frankia* group IIIa, which confirms studies of field collected nodules [38] [9], nodules from inoculated trees contained also *Frankia* group IV. Both introduced strains displayed comparable competitive abilities to each other (Chapter 3 and 4).

6.5. Effect of dried alder leaves on plant growth, N_2-fixation and *Frankia* population

The addition of dried alder leaves to the soil weeks before seedlings were planted resulted in plant growth promotion in non-inoculated as well as in inoculated soils. The increased plant growth was caused by a higher N_2-fixing activity of *Frankia* in the nodules (Chapter 4).

The addition of alder leaves also resulted in population structure changes in the nodules (Chapter 4) and in the soil (Chapter 5). The soil, which was used in these experiments, has an indigenous *Frankia* population consisting of groups IIIa, IIIb and IV. Only group IIIa forms nodules in the field as well as in untreated soil [38] [9]. The addition of alder leaves to the soil resulted in a faster growth of strains belonging to group IV (Chapter 5), so that only group IV could be detected in the nodules (Chapter 4). It has been observed that the addition of host tree (*Casuarina*) cladodes to the soil exhibits a positive effect on the infectious capacity of *Frankia* [39]. This result might be due to either altered soil chemical properties or chemicals present in the green cladode tissue that promoted growth, sporulation, survival or infection capacity of *Frankia* in the soil [39]. Chemicals in host plant parts can have effects on the nodulation capacity as well. For example alder seeds have been reported to contain a flavonoid-like compound which enhances nodulation [40]. In the presented experiment (Chapter 4) however, the change of the *Frankia* population in the nodules was more striking than the change in nodulation capacity. It has been reported that altered environmental conditions lead to a changed population structure in the soil. This is probably caused by the different ability of *Frankia* strains to adapt to changed environmental conditions, either as permanent forms or as vegetative cells [41].

Nevertheless, it is surprising that the addition of alder leaves to a soil that was harvested beneath the canopy of a natural alder stand has such an enormous effect on the indigenous *Frankia* population. Leaves were added before the seedlings were planted and it has been speculated that the competition pressure is lower in soils without host trees [42]. Other possible reasons might be that the added leaves were not derived from the trees growing at the same site from which the soil was harvested, thus their chemical composition might have been slightly different. Additionally, the nitrate concentration in soils with leaves decreased to very low values during the incubation time.

6.6. Conclusion and outlook

An improvement of the *Frankia*-alder symbiosis is possible, either by inoculating pure cultures or by activating the indigenous *Frankia* population with the addition of organic material, e.g. dried alder leaves. The increased growth of *Alnus* after inoculation could even be shown on soils with a high nitrate content or when inoculation took place weeks before alder seedlings were planted. However, the accurate prediction of field results is difficult since the "plant-*Frankia*-environment" system is complex and nodulation as well as the efficiency of the symbiosis can be influenced by various factors [14].

According to Dommergues [43], three points must be kept in mind when trying to improve the symbiosis:

- The best results may be achieved on lands with marginal fertility.
- A method which is effective under certain environmental conditions may fail under other conditions.
- An integrated approach should be chosen where different types of approaches (chemical, biological, physical) assist the most favorable plant-microorganism interaction, e.g. stimulation of the N_2-fixation by adequate soil liming or application of mineral fertilizers such as phosphorus.

This last point is probably not only the most important, but also the most difficult. Additional studies providing further information on the factors influencing the N_2-fixation rate of the *Frankia*-Alnus symbiosis are necessary.

Initially, such studies should focus on the activation of the indigenous *Frankia* population. The factors that are responsible for the change in the *Frankia* population (Chapter 4 and 5) must be identified. Dried alder leaves induced a change by promoting selected strains in the indigenous population. A specific physical or chemical cause for this selection should be determined. In addition, other means to activate the indigenous *Frankia* population should be tested. This could include the addition of organic material, which can be utilized by *Frankia* as C-source, addition of phosphorus or cations, as well as irrigation of dry soils.

Next, the results obtained in the short-term greenhouse-experiments should be evaluated on the same soil in a field setting. Long-term field studies are important and necessary in order to extrapolate laboratory data to real environmental situations. For instance, the *Frankia* strains that were used in the present studies could be inoculated directly into the soil on the field site. Thus, the effect of the inoculation on alder seedlings growing at the site could be assessed. It is also important to know for how many years the inoculated plants maintain an advantage over non-inoculated control plants and whether this time could be extended by any means.

Such long-term studies should also focus on the performance of introduced *Frankia* strains compared to the indigenous population and also the persistence of introduced strains in this soil. Priority should be given to not only identifying the strains showing the best "long-term-performance" regarding persistence in the soil, infectious capacity, and N_2-fixation rates, but also to determining the optimal environmental conditions for the activity of these strains.

6.7. References

1. **Dommergues, Y.R.** 1996. Contribution of actinorhizal plants to tropical soil productivity and rehabilitation. Soil Biology and Biochemistry 29(5/6): 931-941.

2. **Arnone, J.A., S.J. Kohls and D.D. Baker.** 1994. Nitrate effects on nodulation and nitrogenase activity of actinorhizal *Casuarina* studied in split-root systems. Soil Biology and Biochemistry 26(5): 599-606.

3. **Van Dijk, C. and A. Sluimer.** 1994. Resistance to an ineffective *Frankia* strain type in *Alnus glutinosa* (L.) Gaertn. New Phytologist 128: 497-504.

4. **Kohls, S.J. and D.D. Baker.** 1989. Effects of substrate nitrate concentration on symbiotic nodule formation in actinorhizal plants. Plant and Soil 118: 171-179.

5. **Chatarpaul, L., P. Chakravarty and P. Subramaniam.** 1989. Studies in tetrapartite symbiosis. Plant and Soil 118: 145-150.

6. **Hahn, D., M.J.C. Starrenburg and A.D.L. Akkermans.** 1990. Growth increment of *Alnus glutinosa* upon dual inoculation with effective and ineffective *Frankia* strains. Plant and Soil 122: 121-127.

7. **Dawson, J.O., D.G. Kowalski and P.J. Dart.** 1989. Variation with soil depth, topographic position and host species in the capacity of soils from an Australian locale to nodulate *Casuarina* and *Allocasuarina* seedlings. Plant and Soil 118: 1-11.

8. **Kurdali, F. and A.M. Domenach.** 1991. Evaluation of nodulation capacity in *Alnus glutinosa* seedlings grown in soil sampled from various depths. Acta Oecologica 12: 397-401.

9. **Zepp, K., D. Hahn and J. Zeyer.** 1997. *In situ* analysis of introduced and indigenous *Frankia* populations in soil and root nodules obtained on *Alnus glutinosa*. Soil Biology and Biochemistry 29(9/10): 1595-1600.

10. **Huss-Danell, K., P. Lundquist and H. Ohlsson.** 1992. N_2 fixation in a young *Alnus incana* stand, based on seasonal and diurnal variation in

whole plant nitrogenase activity. Canadian Journal of Botany 70: 1537-1544.

11. **Pratt, S.D., A.S. Konopka, M.A. Murry, F.W. Ewers and S.D. Davis**. 1997. Influence of soil moisture on the nodulation of post fire seedlings of *Ceanothus* spp. growing in the Santa Monica Mountains of Southern California. Physiologia Plantarum 99: 673-679.

12. **Zitzer, S.F. and J.O. Dawson**. 1992. Soil properties and actinorhizal vegetation influence nodulation of *Alnus glutinosa* and *Elaeagnus angustifolia* by *Frankia*. Plant and Soil 140: 197-204.

13. **Sayed, W.F., C.T. Wheeler, H.H. Zahran and A.A.M. Shoreit**. 1997. Effect of temperature and soil moisture on the survival and symbiotic effectivness of *Frankia* spp. Biology and Fertility of Soils 25: 349-353.

14. **Smolander, A.** 1990. *Frankia* populations in soils under different tree species - with special emphasis on soil under *Betula pendula*. Plant and Soil 121: 1-10.

15. **Dawson, J.O. and J.W. Van Sambeck**. 1993. Interplanting woody nurse crops promotes differential growth of black walnut saplings. In: Proceedings, 9th Central Hardwood Forest Conference, Eds.: A.R. Gillespie, P.E. Pope, G. Rink, U.S. Department of Agriculture, Forest Service, North Central Forest Experiment Station: West Lafayette: 455-463.

16. **Huss-Danell, K.** 1986. Growth and production of leaf litter nitrogen by *Alnus incana* in response to liming and fertilization on degenerated forest soil. Canadian Journal of Forest Research 16: 847-853.

17. **Arveby, A.S. and K. Huss-Danell**. 1988. Presence and dispersal of infective *Frankia* in peat and meadow soils in Sweden. Biology and Fertility of Soils 6(1): 38-44.

18. **Amann, R.I.** 1995. Fluorescently labeled, rRNA-targeted oligonucleotide probes in the study of microbial ecology. Molecular Ecology 4: 543-554.

19. **Bosco, M., M.P. Fernandez, P. Simonet, R. Materassi and P. Normand**. 1992. Evidence that some *Frankia* spp. strains are able to

cross boundaries between *Alnus* and *Elaeagnus* host specificity groups. Applied and Environmental Microbiology 58(5): 1569-1576.

20. **Cournoyer, B. and P. Normand**. 1994. Characterization of a spontaneous thiostrepton-resistant *Frankia alni* infective isolate using PCR-RFLP of *nif* and *gln*II genes. Soil Biology and Biochemistry 5: 553-559.

21. **Hahn, D., M. Dorsch, E. Stackebrandt and A.D.L. Akkermans**. 1989. Synthetic oligonucleotide probes for identification of *Frankia* strains. Plant and Soil 118: 211-219.

22. **Hahn, D., R. Kester, M.J.C. Starrenburg and A.D.L. Akkermans**. 1990. Extraction of ribosomal RNA from soil for detection of *Frankia* with oligonucleotide probes. Archives of Microbiology 154: 329-335.

23. **Hönerlage, W., D. Hahn, K. Zepp, J. Zeyer and P. Normand**. 1994. A hypervariable 23S rRNA region provides a discriminating target for specific characterization of uncultured and cultured *Frankia*. Systematic and Applied Microbiology 17: 433-443.

24. **Mirza, M.S., S. Hameed and A.D.L. Akkermans**. 1994. Genetic diversity of *Datisca cannabina*-compatible *Frankia* strains as determined by sequence analysis of the PCR-amplified 16S rRNA gene. Applied and Environmental Microbiology 60(7): 2371-2376.

25. **Simonet, P., P. Normand, M. A and R. Bardin.** 1990. Identification of *Frankia* strains in nodules by hybridization of polymerase chain reaction products with strain specific oligonucleotide probes. Archives of Microbiology 153: 235-240.

26. **Fried, M. and V. Middelboe**. 1977. Measurement of amount of nitrogen fixed by a legume crop (Soybean). Plant-Soil 47(3): 713-715.

27. **McNeill, A.M., R.C. Hood and M. Wood**. 1994. Direct measurement of nitrogen fixation by *Trifolium repens* L. and *Alnus glutinosa* L. using $^{15}N_2$. Journal of Experimental Botany 45(275): 749-755.

28. **Witty, J.F.** 1983. Estimating N_2-fixation in the field using ^{15}N-labelled fertilizer: Some problems and solutions. Soil Biology and Biochemistry 15(6): 631-639.

29. **Sougoufara, B., S.K.A. Danso, H.G. Diem and Y.R. Dommergues.** 1990. Estimating N_2 fixation and N derived from soil by *Casuarina equisetifolia* using labeled ^{15}N fertilizer: some problems and solutions. Soil Biology and Biochemistry 22: 695-701.

30. **Ledgard, S.F., J.R. Simpson, J.R. Freney and F.J. Bergersen.** 1985. Field evaluation of ^{15}N techniques for estimating nitrogen fixation in legume-grass associations. Australian Journal of Agricultural Research 36: 247-258.

31. **Ledgard, S.F., J.R. Simpson, J.R. Freney and F.J. Bergersen.** 1985. Effect of reference plant on estimation of nitrogen fixation by sub-clover using ^{15}N methods. Australian Journal of Agricultural Research 36: 663-676.

32. **Mansour, S.R. and D.D. Baker.** 1994. Selection trials for effective N_2-fixing *Casuarina-Frankia* combinations in Egypt. Soil Biology and Biochemistry 26(5): 655-658.

33. **Sellstedt, A., L. Stahl, U. Mattsson, K. Jonsson and P. Högberg.** 1993. Can the ^{15}N dilution technique be used to study N_2 fixation in tropical tree symbioses as affected by water deficit? Journal of Experimental Botany 44(269): 1749-1755.

34. **Steele, D.B., K. Ramirez and M.D. Stowers.** 1989. Host plant growth response to inoculation with *Frankia*. Plant and Soil 118: 139-143.

35. **Strukova, S., M. Vosatka and J. Pokorny.** 1996. Root symbioses of *Alnus glutinosa* (L.) Gaertn. and their possible role in alder decline: a preliminary study. Folia Geobotanic and Phytotaxonomy 31: 53-162.

36. **Myrold, D.D. and K. Huss-Danell.** 1994. Population dynamics of *Alnus* infective *Frankia* in a forest soil with and without host trees. Soil Biology and Biochemistry 26: 533-540.

37. **Maunuksela, L., K. Zepp, T. Koivula, J. Zeyer, K. Haahtela and D. Hahn**. 1999. Analysis of *Frankia* in three soils devoid of actinorhizal plants. FEMS Microbiology Ecology 28: 11-21.

38. **Zepp, K., D. Hahn and J. Zeyer**. 1997. Evaluation of a 23S rRNA insertion as target for the analysis of uncultured *Frankia* populations in root nodules of alders by whole cell hybridization. Systematic and Applied Microbiology 20: 124-132.

39. **Zimpfer, J.F., G.J. Kennedy, C.A. Smyth, J. Hamelin, E. Navarro and J.O. Dawson**. 1999. Localization of *Casuarina*-infective *Frankia* near *Casuarina cunninghamiana* trees in Jamaica. Canadian Journal of Botany 77(9): 1248-1256.

40. **Benoit, L.F. and A.M. Berry**. 1997. Flavonoid-like compounds from seeds of red alder (*Alnus rubra*) influence host nodulation by *Frankia* (Actinomycetales). Physiologia Plantarum 99: 588-593.

41. **Nalin, R., P. Normand and A.M. Domenach**. 1997. Distribution and N_2-fixing activity of *Frankia*-strains in relation to soil depth. Physiologia Plantarum 99: 732-738.

42. **McEwan, N.R., E.M.O. Gould, E. Lumini, J.D. McNeill and C.T. Wheeler**. 1998. Competitivity and persistence of Frankia in a managed environment. In: Nitrogen fixation with non-legumes. Proceedings of the 7th international symposium on nitrogen fixation with non-legumes, Eds.: A. Malik, M.S. Mirza, and J.K. Ladha, Kluwer Academic Publishers, Dordrecht: 315-326.

43. **Dommergues, Y.R.** 1978. Impact on soil management and plant growth. In: Interactions between non-pathogenic soil microorganisms and plants, Eds.: Y.R. Dommergues and S.V. Krupa, Elsevier Scientific Publishing Company, Amsterdam: 443-458.

Seite Leer /
Blank leaf

Seite Leer /
Blank leaf

Acknowledgements

I am grateful to Prof. Dr. Josef Zeyer for giving me the opportunity to carry out this thesis, encouraging my work, providing valuable suggestions, and giving me the chance to meet other researchers from many different countries.

I would also like to thank Prof. Dr. Anton Hartmann and Dr. Rolf Siegwolf for agreeing to co-examine my thesis. I would like to particularly thank my supervisor Dr. Dittmar Hahn. Without his professional expertise this thesis would not have been possible. I appreciated his constant support and valuable suggestions. Dr. Franco Widmer and Dr. Martin Schroth provided me with helpful discussions for which I am appreciative.

Part of the research for thesis was carried out in cooperation with other institutions. Dr. Rolf Siegwolf and Dr. Matthias Saurer from the Paul Scherrer Institute (PSI), with whom it has been a pleasure to work, gave me insight about working with stable isotopes. Prof. Dr. Jeff Dawson, Jeff Zimpfer and Isabelle Latorre from the University of Illinois at Urbana-Champaign (UIUC) made me feel welcome, and I am grateful for having found myself in such a hospitable atmosphere while working in a foreign country. I would also like to thank Dr. Anton Burkhart for providing room in the greenhouses at the Swiss Federal Institute for Forest, Snow and Landscape Research (WSL).

My research was aided by grants from the Swiss National Science Foundation (Priority Program Biotechnology), and the Swiss Federal Office of Environment, Forests and Landscape (BUWAL).

My outlines were read and criticized constructively by several people and they all deserve special thanks for their helpful comments on the manuscript. In particular I would like to mention Prof. Dr. Josef Zeyer, Dr. Dittmar Hahn and Dr. William Von Sigler.

I would like to thank the people at the Institute of Terrestrial Ecology for showing me a good time, in particular Dr. Christof Bolliger, Dr. Antonis Chatzinotas, Dr. Lukas Kohli, Dr. Annatina Zarda and Dr. Boris Zarda. Several people helped me to harvest hundreds of plants and thousands of nodules, and I am appreciative for their assistance. Additionally, I am grateful to Martin Thullner and Helmut Bürgmann for their computer support.

I thank particularly all the people who gave me not only moral support but also shared many "non-*Frankia*-projects" with me, especially my family and Markus.

Seite Leer /
Blank leaf

Curriculum vitae

1970	Born on 2 May in Munich, Germany
1976-1980	Primary school in Munich, Germany
1980-1989	Gymnasium in Munich, Germany
1989-1995	Studies of Biology at the Ludwig-Maximilians-University in Munich, Germany
1994	Practical training at the Institut Pasteur, Paris, France
1995-1996	Research assistant at the gsf, Munich, Germany
1996-2000	PhD at the Institute of Terrestrial Ecology, Soil Biology, ETH, Zürich, Switzerland
1997	Research at the University of Illinois at Urbana-Champaign, USA