LIFE IN SOIL BY THE ACTINORHIZAL ROOT NODULE ENDOPHYTE *FRANKIA*. A REVIEW

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ABSTRACT

Frankia is a genus of soil actinomycetes famous for its ability to form N_2-fixing root nodule symbioses with actinorhizal plants. Although *Frankia* strains diplay a high diversity in terms of ecological niches in soil, current knowledge about *Frankia* is dominated by its life as an endophyte in root nodules. Increased use of molecular methods has refined and expanded insights into endophyte-host specificities and *Frankia* phylogeny. This review has focus on *Frankia* as a soil organism, including its part of microbial consortia, and how to study *Frankia* in soil. We highlight the use of nodulation tests and molecular methods to reveal population size and genetic diversity of *Frankia* in soil and discuss how autoregulation of nodulation and interactions with other soil microorganisms may influence the results. A comprehensive record of published interactions between *Frankia* and other soil microbes is summarized.

Keywords Actinomycetes, *Frankia* detection methods, *Frankia* ecology, N_2 fixation, plant-microbe interaction, symbiosis

Abbreviations GU, genomic units; MPN, most probable number; NC, nodulation capacity; NT nodulation test; NU, nodulating units; PCR, polymerase chain reaction

1. Introduction

Frankia is a genus of soil actinomycetes in the family Frankiaceae occurring also in symbiosis with certain angiosperms. The actinomycete *Frankia* is defined as the N_2-fixing microsymbiont of actinorhizal plants (Wall 2000) (for morphological description, see section 2). Following this definition and Koch's postulates, *Frankia* should be isolated from root nodules of actinorhizal plants and *Frankia* isolates should be able to induce nodules on the actinorhizal plants (Figures 1 and 2). Early studies of symbiotic *Frankia*, including the uncertainties about the identity of the inhabitant of actinorhizal root nodules, are reviewed by Quispel (1990). *Frankia* as a generic name was proposed by Brunchorst (1886-1888) for the microorganism in nodules of *Alnus* and Elaeagnaceae. Knowledge about *Frankia* was previously restricted to its symbiotic stage because of difficulties in isolating *Frankia* into pure cultures. Pommer (1959) most likely made the first successful and well described isolation from *Alnus glutinosa* nodules, but his cultures were unfortunately lost. Twenty years later Callaham et al. (1978) reported isolation of *Frankia* CpI1 from *Comptonia peregrina*. Numerous isolates of *Frankia* then became available from many but not all actinorhizal plant species. When *Frankia* isolates are not available crushed root nodules or soil must be used as inoculum. When *Frankia* in soil is investigated via a trap plant assay, the *Frankia* being studied is the strain(s) able to nodulate the plants.

The *Frankia* species previously proposed by Becking (1970) were based on cross-inoculation experiments with crushed nodules, and were abandoned as isolates became available. Instead, only strain designations were proposed. They consist of a three letter acronym for the research group that obtained the isolate and a strain number of up to 10 numerical digits where the first two represent the host genus and the next two represent the host species from where the isolate was obtained (Lechevalier 1983). This nomenclature has not been fully adopted and *Frankia* strains are often designated simply with letters for genus and species of corresponding original host plant and an isolate number, e.g. ArI3 for isolate number 3 from *Alnus rubra* (Berry and Torrey 1979).

Molecular taxonomic procedures are now used as alternatives to techniques limited by successful isolation of *Frankia*. A comparative sequence analysis of 16S ribosomal DNA led to the emendation of family Frankiaceae to contain only the genus *Frankia* with four main subdivisions or clusters (Normand et al. 1996). Several genes including *glnII*, intergenic spacers of 16S-23S rDNA, *nif*H-D and *nif*D-K operons have afterwards been successfully used to confirm and describe diversity within these clusters (Hahn et al. 1999; Hahn 2008) as specific targets for characterization of isolates as well as for identification of uncultured endophytes in root nodules. *Frankia* groups can be described as: Cluster 1: a group of strains comprising *Frankia alni* and other typical *Frankia* strains infecting *Alnus, Casuarina* and *Myrica* host groups; Cluster 2: unculturable *Frankia* endophytes from nodules of *Dryas, Coriaria* and *Datisca* including also *Ceanothus* ; Cluster 3: strains of Elaeagnaceae and most Rhamnaceae excluding *Ceanothus* strains ; Cluster 4: atypical non-N_2-fixing strains or strains that are not able to reinfect the original host but have been isolated from actinorhizal nodules .

These new procedures reveal much about the genetic diversity and distribution of *Frankia*, and have refined and expanded knowledge about endophyte-host specificities (see Table 1). Due to the complexity and diversity of *Frankia*, polyphasic taxonomy approaches seem to be more appropriate, integrating information retrieved by a wide range of techniques on different levels of taxonomic resolution (Hahn 2008). Currently only clusters or groups of *Frankia* are being considered based on phylogenetic analysis of strain gene sequences (Benson and Dawson 2007; Normand et al. 1996).

Actinorhizal plants comprise some 200 plant species belonging to 25 genera in eight families (Table 1) (Figure 1 A, B). These plants belong to the Eurosid I clade where legumes and *Parasponia* are also placed (Soltis et al. 1995) suggesting a common evolutionary origin of root nodule symbioses. Actinorhizal symbioses are not obligate for the host. For example, *Alnus* species can be grown in the greenhouse without nodules if provided with nitrate or ammonium (Hiltner 1895; Sellstedt and Huss-

Danell 1986), but there are no reports of non-nodulated *Alnus* in the field. For *Frankia*, symbiosis is not obligatory in cases where pure isolate cultures have been obtained from nodules such as *Frankia* belonging to clusters 1 and 3 (Benson and Dawson 2007), which nodulate genera in the families Betulaceae, Casuarinaceae, Myricaceae, Elaeagnaceae and Rhamnaceae – except *Ceanothus*. However, no isolate has been cultivated in vitro for *Frankia* belonging to cluster 2. *Frankia* cluster 2 (Benson and Dawson 2007) includes *Frankia* that nodulate actinorhizal plants belonging to the Rosaceae, Coriariaceae and Datiscaceae families, and to the genus *Ceanothus* (Table 1). If these *Frankia* really are obligate symbionts, then the obvious question is: how are roots infected to yield root nodules? It seems more likely that their cultivation *in vitro* needs a nutrient or growth factor which has not yet been identified. Work on the genome sequence of the endosymbiont of *Datisca glomerata* is in progress (K. Pawlowski, personal communication) and will likely help to solve this matter.

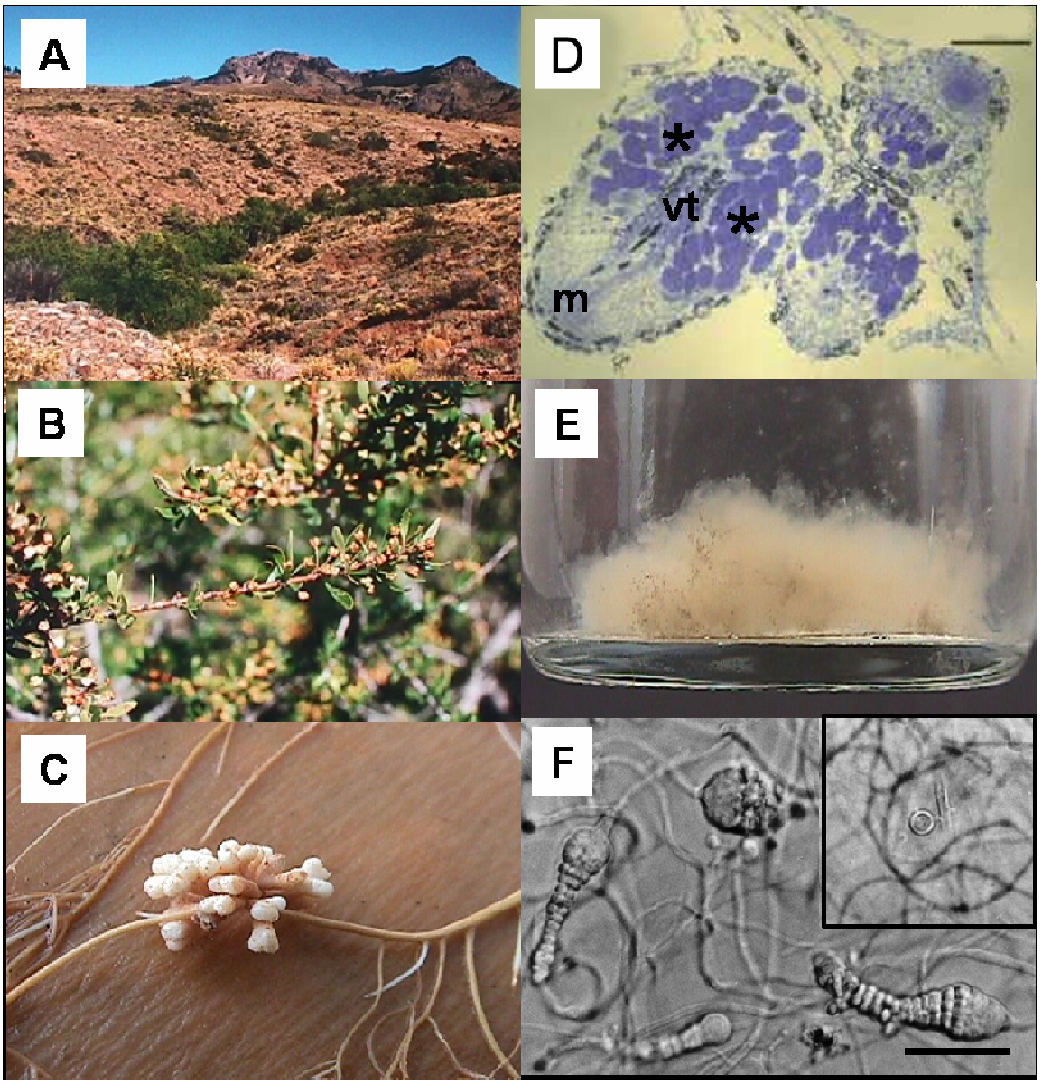

Figure 1. Actinorhizal symbioses as examplified from Patagonia, Argentina. A) *Discaria chacaye* shrubs growing along a river in northwestern Patagonia; B) *Discaria trinervis* shoot with mature fruits; C) *Discaria trinervis* multilobed nodule; D) *Discaria trinervis* mature nodule in longitudinal section showing characteristic central vascular tissue (vt), apical meristem (m) and infected cells full of vesicle clusters stained blue (*); E) liquid culture of *Frankia* strain DcI45 isolated from *Discaria chacaye* root nodules; F) *Frankia* strain DaI1 isolated form *Dicsaria articulata* showing characteristic hyphae, multilocular sporangia, spores and a spherical vesicle (inset).

Is *Frankia* then synonymous with actinorhizal endophytes? Historically *Frankia* was defined as the microsymbiont of actinorhizal nodules and if a positive nodulation was obtained in a plant inoculated with soil, the interpretation was that *Frankia* was trapped. This kind of interpretation should be revised. Many strains have been isolated from actinorhizal nodules but do not fulfill criteria commonly perceived as defining *Frankia* as they were lacking vesicles, sporangia, or N_2 fixation ability, or the capacity to induce infections and nodules in actinorhizal roots. Such strains were designated as atypical "non-infective on their original host, devoid of N_2-fixing ability, not capable to form vesicles" (Gauthier et al. 1999; Valdés La Hens 2007; Van Dijk and Sluimer-Stolk 1990; Van Dijk and Sluimer 1994). Due to this high strain diversity, Akkermans and Hirsch (1997) revised the term 'atypical' and proposed a new strain designation which indicates nodulation and N_2 fixation abilities (Nod^+/Nod^-, Fix^+/Fix^-). To add more uncertainties to the definition of *Frankia*, non-*Frankia* N_2-fixing nodule endophytes were also detected (Ghodhbane et al. 2010; Gtari et al. 2007a; Mirza et al. 1994; Trujillo et al. 2006; Valdés et al. 2005; Valdés La Hens 2007) (Figure 2 notes 3, 6).

Sequencing of the first three *Frankia* genomes opened new opportunities to understand *Frankia* (Normand et al. 2007a, 2007b), and prompted the scientific community to search for additional genomic information (Table 2). When we include very specific and restrictive strains, and examples of non-*Frankia* endophytes, the possibility to compare genomes from typical *Frankia* isolates in different cross-inoculation groups will help us to better understand what genetic information is necessary to induce and develop, in joint action with the plant, a symbiotic actinorhizal nodule. Genomic analysis and comparisons will help us understand the physiology of *Frankia* in its symbiotic and its free-living states. Hopefully this will facilitate finding the requirements for *in vitro* growth in those cases where isolation of *Frankia* from nodules so far has failed. In order to understand the potential of *Frankia* to synthesize and respond to plant signals related to the interaction and recognition with the host root, *Frankia* genomes are also subject to proteomics experiments, *i.e.*, to search for genes that are differentially expressed in nodules or in the presence or in the presence of root exudates, in order to investigate the communication system and the genetic basis of molecular interactions in actinorhizal symbioses (Alloisio et al. 2007; Bagnarol et al. 2007; Mastronunzio and Benson 2010).

There is much information about *Frankia* in symbiosis and *Frankia* in culture, but less focus has been given to *Frankia* as a soil organism (Dawson 2008; Hahn et al. 1999; Valdés 2008). We highlight the following aspects of *Frankia* in soil: morphology, isolation from soil, N_2 fixation in soil, nodulation tests, DNA methods, occurrence and dispersal of *Frankia*, and interactions between *Frankia* and other soil organisms.

2. Morphology of *Frankia*

In culture, three morphological forms are characteristic for *Frankia*: hyphae (or filaments), spores, and vesicles (Figure 1 E, F). Hyphae are typically 0.5 µm thick, septate and branched while vesicles are spherical, about 1-5 µm in diameter and septate (Newcomb and Wood 1987). Vesicles are typically formed under N limited conditions and are the site of the N_2-fixing enzyme dinitrogenase (Meesters 1987; Tjepkema et al. 1980). A striking feature of the vesicles is the surrounding envelope which consists of multiple layers of bacterial steroid lipids, hopanoids (Berry et al. 1993), which presumably assist in regulation of oxygen tension near dinitrogenase. The envelope is continuous, but much thinner around hyphae. Multilocular sporangia with spores are formed terminally or in an intercalary position on the hyphae (Lechevalier and Lechevalier 1984). Spores will germinate into hyphae.

In root nodules (Figure 2 C, D), hyphae are always present and vesicles are found in nodules of all studied genera except *Casuarina* and *Allocasuarina*. Vesicle shape and size, presence or absence of septa in vesicles, and the spatial distribution of vesicles within an infected plant cell differ among symbioses and are determined by the host (Huss-Danell 1997). Vesicles are the site of dinitrogenase (Huss-Danell and Bergman 1990). However, in *Casuarina* and *Allocasuarina* dinitrogenase must be localized in hyphae. *Frankia* may or may not form spores within nodules, and according to spore formation nodules are designated as Sp+ or Sp-, respectively (Van Dijk and Merkus 1976). Presence of spores has been studied mainly in *Alnus*, *Comptonia* and *Myrica* (Schwintzer 1990; Torrey 1987; Van Dijk and Merkus 1976; Van Dijk 1978) and depends on the *Frankia* strain and environmental factors (Simonet et al. 1994; Zepp et al. 1997a).

We are not aware of any published pictures of *Frankia* occurring naturally in soil, but we assume that under appropriate conditions *Frankia* will show the same appearance in soil as in culture, *i.e*. hyphae,

sporangia with spores and, under low environmental N, vesicles. If so, we can expect that the spores are important in dispersal of *Frankia,* carried for instance by water or by soil fauna. *Frankia* has been observed in soil samples in microcosms by use of *in situ* hybridization with specific probes (Hahn et al. 1999; Mirza et al. 2007). With this method *Frankia* growth was shown in litter-amended soil and in rhizosphere of non-actinorhizal plants. *Frankia* growth was seen as an increase in cell numbers and hyphal length (Mirza et al. 2009). Although neither vesicles nor sporangia have yet been observed in that particular assay, these molecular techniques promise to be very useful to future study of *Frankia* in soil.

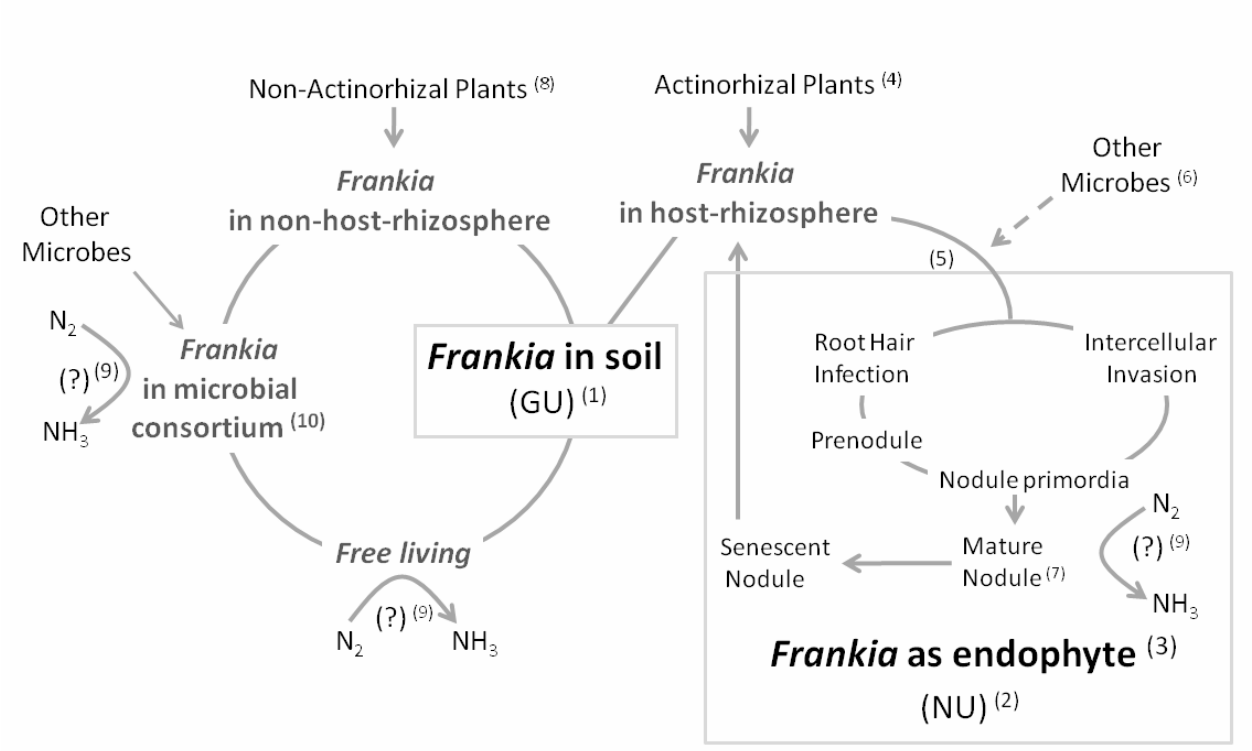

Figure 2. Schematic model of main ecological niches and interactions of *Frankia* in soil (1) GU, Genomic Units, refer to measurements of *Frankia* using PCR methods and environmental DNA samples that can be obtained from any *Frankia* ecological niche; (2) NU, Nodulating Units, refer to measurements of *Frankia* using trap plant assays. This method allows detection only of infective *Frankia* in soil samples and has some limitations considering symbiotic specificity with corresponding host-plant (see the text); (3) *Frankia* endophytes include atypical *Frankia* and non-*Frankia* actinomycetes that have been isolated from actinorhizal nodules; (4) Actinorhizal Plants refers here to the corresponding host plant for a certain *Frankia* strain that can fulfil symbiotic recognition and induce N_2-fixing root nodules as the final expression of actinorhizal symbiosis; (5) Chemical communication with signal exchange and recognition between the plant and the bacteria are needed to allow infection in order for *Frankia* to enter an endophytic phase of life; (6) Helper bacteria and actinomycetes have been described to be involved in early interactions enhancing infection and nodulation (see the text); (7) Mature nodule can be effective and fix N_2 if *Frankia* differentiates into vesicles (all genera except *Casuarina* and *Allocasuarina*) and appropriately expresses dinitrogenase; otherwise *Frankia* can induce ineffective, non N_2-fixing nodules; (8) Non-actinorhizal plants refers either to plants that never form symbiosis with *Frankia* or to actinorhizal plants that can not fulfil recognition steps with *Frankia* and, consequently, infection and nodulation is not possible; (9) *Frankia* is able to fix N_2 in free-living state in laboratory culture, thus it is supposed to be able to do so also in free-living state in nature but there is no proof of this function in nature; (10) *Frankia* can produce some compounds recognized to be involved in bacteria-bacteria interactions such as quorum-sensing, but almost nothing is known about *Frankia* interaction with other microorganisms and microbial consortium formation.

Table 1. Presence of *Frankia* in soils under host plants or lacking host plants and characterization of strains in nodules of the trap plants (T), field plants (F), or *Frankia* DNA extracted from soils (S). *Frankia* clusters, major strain groups having different phylogeny and host specificity (Benson and Dawson 2007). Numbers refer to nodulating units per g (g) or per cm^3 (c) of soil, determined by any of the following methods: most probable number (MPN), nodulation capacity (NC) and least square estimates (LS), or to mean number of nodules per plant (np) determined by a nodulation test (NT) in air-dried (d) or fresh (f) soil samples. Only selected examples of nodulating units are given. Molecular characterization methods have been performed on DNA samples obtained from field nodules (F) or nodules after trap plant assay (T).

Host family, Genus	*Frankia* cluster	Locality	Quantification method	*Frankia* nodulating units in soil		Molecular characterization method	Reference
				under host plant	lacking host plant		
Betulaceae							
Alnus	1	Finland	NC, f	299 c	nd [a] - 2940 c [b, c]		Smolander and Sundman 1987
		Finland	NC		2267-3160 g	T, S. 23S rRNA sequencing, rep-PCR, in situ hybridization nested PCR	Maunuksela et al. 1999
		Sweden	MPN, f	nd	5- 400 g [d]		Huss-Danell and Myrold 1994
		Sweden	NC, f	nd	10 - 380 g [d]		Huss-Danell and Myrold 1994
		The Netherlands	NC, f	404 - 28400 c			Van Dijk 1984
		Canada	NC, f	14 -19 c	0 -12 c		Markham and Chanway 1996
		The Netherlands	NC, f	0 - 49 g		T. 16S rDNA gene sequencing	Wolters et al. 1997a; 1997b
		USA	LS, d	Up to 9 c [e]	Up to 5 c [e]	T. PCR-RFLP of 16S/23S IGS rRNA	McCray Batzli et al. 2004; Huguet et al. 2004
		USA	MPN, na [f]	0.7-393 g	nd		Martin et al. 2003
		USA	LS, d		0 - 7 c ; 97 - 238 c [c]		Paschke et al. 1994
		Hawaii	MPN, d	nd	nd		Burleigh and Dawson 1994a
		Costa Rica	NT, f		na		Paschke and Dawson 1992
		Argentina		Qualitative data			Tortosa and Medan 1989
		Tunisia		Qualitative data [g]	Qualitative data	T. rDNA ARDRA, 16S rRNA sequencing	Gtari et al. 2007b
Casuarinaceae							
Allocasuarina	1	Australia	NT, na		0.1 np		Dawson et al. 1989

Table 1 (continued)

Host family, Genus	*Frankia* cluster	Locality	Quantification method	*Frankia* nodulating units in soil		Molecular characterization method	Reference
				under host plant	lacking host plant		
Casuarina	1	Jamaica	LS, d	14 - 8548 g	nd	T. PCR-RFLP	Zimpfer et al. 1999
		Jamaica	MPN, d		nd		Zimpfer et al. 1997
		Jamaica	LS, d		nd		Zimpfer et al. 1997
		Hawaii	MPN, d	nd	nd		Burleigh and Dawson 1994a
		Australia	NT, na	0.4 – 1.6 np			Dawson et al. 1989
		Tunisia		Qualitative data		T. rDNA ARDRA, 16S rRNA sequencing	Gtari et al. 2007b
Ceuthostoma [h]		Philippines, Borneo, N.Guinea		Qualitative data(?)			See note [h]
Gymnostoma	3	New Caledonia		Qualitative data		F. PCR-RFLP rrs-rrl (16S-23S) IGS	Navarro et al. 1999
Myricaceae							
Comptonia	1	Nova Scotia		Qualitative data			Bond 1976
		USA, Portugal		Qualitative data		F. 16S rRNA gene partial sequences	Clawson and Benson 1999
Myrica	1	USA	LS, d		1-13 c		Paschke et al. 1994
		USA	LS, d			T. PCR-RFLP of 16S/23S IGS rRNA	McCray Batzli et al. 2004; Huguet et al. 2004
		Hawaii	MPN, d	6.7-123.7 c	0 - 0.22 c		Burleigh and Dawson 1994a
		Jamaica	LS, d		2057 - 20131 g	T. PCR-RFLP	Zimpfer et al. 1999
		Jamaica	MPN, d		nd - 1379 c		Zimpfer et al. 1997
		Jamaica	LS, d		nd - 1843 c		Zimpfer et al. 1997
Morella [i]	1, 3	Africa, Europe, Asia, North America, and South America		Qualitative data		T. sequence analyses of *nif*H gene fragments	Welsh et al. 2009
	1, 3	USA, UK		Qualitative data		F. 16S rRNA gene partial sequences	Clawson and Benson 1999
Elaeagnaceae							
Elaeagnus	3	France	NC, f		87.2 c	T. PCR-RFLP nifD-K IGS	Nalin et al. 1997
		France			Qualitative data	S. nifD-K IGS, hybridization and sequencing	Nalin et al. 1999

Table 1 (continued)

Host family, Genus	*Frankia* cluster	Locality	Quantification method	*Frankia* nodulating units in soil		Molecular characterization method	Reference
				under host plant	lacking host plant		
		USA	LS, d		11 - 125 c		Paschke et al. 1994
		Costa Rica	NT, f		Qualitative data		Paschke and Dawson 1992
		Tunisia		Qualitative data	Qualitative data	T. 16S rRNA and GLnII sequencing, rep-PCR, tDNA-PCR-SSCP	Gtari et al. 2004
		Tunisia		Qualitative data	Qualitative data	T. rDNA ARDRA, 16S rRNA sequencing	Gtari et al. 2007b
Hippophaë	3	The Netherlands	NT, na	10 - 181 np	4 - 20 np		Oremus 1980
Shepherdia	3	USA	LS, d	Up to 20 c	7 c	T. PCR-RFLP of 16S/23S IGS rRNA	McCray Batzli et al. 2004; Huguet et al. 2004
Rhamnaceae							
Adolphia [j]	?	Mexico		Qualitative data			Cruz-Cisneros and Valdés 1991
Ceanothus	2	USA	MPN, f [k]	0.9-2.4 [e] g	0.2 - 0.3 g [e]	T. Rep-PCR	Jeong and Myrold 2001
		USA	NC, f [k]	3.6-5.2 [e] g	0.2 - 0.4 g [e]	T. Rep-PCR	Jeong and Myrold 2001
Colletia	3	Argentina		Qualitative data			Tortosa and Medan 1989
Discaria [l]	3	Argentina	MPN, f	8.5- 98.0 g	0-340.0 g		Chaia et al. 2006a
		New Zealand		Qualitative data			Newcomb and Pankhurst 1982
Kentrothamnus	3	Argentina		Qualitative data			Tortosa and Medan 1989
Retanilla	3	Chile		Qualitative data			Silvester et al. 1985
Talguenea	3 [m]	Chile		Qualitative data			Silvester et al. 1985
Trevoa	3	Argentina		Qualitative data			Tortosa and Medan 1989
Rosaceae							
Cercocarpus	2	USA		Qualitative data		F. 16S rRNA gene and glnA and ITS DNA sequencing	Vanden Heuvel et al. 2004
		USA		Qualitative data			Baker and O'Keefe 1984
Chamaebatia	2	USA		Qualitative data		F. 16S rRNA gene and glnA and ITS DNA sequencing	Vanden Heuvel et al. 2004
Cowania	2 [n]	USA	NT, d	na			Righetti et al. 1986
Dryas	2	Canada		Qualitative data			Kohls et al. 1994

Table 1 (continued)

Host family, Genus	*Frankia* cluster	Locality	Quantification method	*Frankia* nodulating units in soil		Molecular characterization method	Reference
				under host plant	lacking host plant		
Purshia	2	USA		Qualitative data		F. 16S rRNA gene and glnA and ITS DNA sequencing	Vanden Heuvel et al. 2004
		USA	NT, d	0 - 23 np [o]			Righetti et al. 1986
Coriariaceae							
Coriaria	2	New Zealand		Qualitative data		T. 16S rRNA gene partial sequences	Clawson et al. 1997
		Argentina		Qualitative data			Medan and Tortosa 1981
		Mexico		Qualitative data			Cruz-Cisneros and Valdés 1990
		Pakistan		Qualitative data			Chaudhary et al. 1985
Datiscaceae							
Datisca	2	USA		Qualitative data		F. 16S rRNA gene and glnA and ITS DNA sequencing	Vanden Heuvel et al. 2004
		Pakistan		Qualitative data			Chaudhary et al. 1985

[a] nd: not detected
[b] Range of nodulation units among sites
[c] Soil samples collected under *Betula* spp.
[d] Range of nodulation units among trap plants belonging to the same genus
[e] Estimated mean values from a graph.
[f] na: information not available.
[g] Qualitative data, *Frankia* presence in soils inferred by the occurrence of nodulated plants under natural conditions.
[h] Listed as nodulated in several review articles but primary article not found.
[i] For several species the genus name *Myrica* has been replaced with genus name *Morella.*
[j] *Adolphia infesta* (H.B.K.) : syn: *Ceanothus infestus* (H.B.K.)
[k] Soil samples stored at -20ºC after sampling.
[l] *Discaria trinervis* has been renamed as *Ochetophila trinervis* (Kellermann et al. 2005)
[m] from Hahn 2008
[n] from Dawson 2008
[o] Soils down to 40 cm depth.

Table 2: Diversity of *Frankia* in soil studied in field collected nodules (N) or in isolates from nodules (I).

Host plant	Location	Methods	Characterization of actinorhizal nodule endophytes or isolates.	Reference
Ceanothus americanus	USA	N. RFLP and hybridization of *nif*DH	There is genetic diversity among geographical locations and among plants in a single location.	Baker and Mullin 1994
Discaria toumatou	New Zealand	N. 16S rRNA gene partial sequences	Unique sequence that places the strains in a lineage close to endophytes of Elaeagnaceae.	Benson et al. 1996
Ceanothus griseous *Purshia tridentata*	New Zealand	N. 16S rRNA gene partial sequences	Identical sequences to those for the endophyte of *Dryas drummondii*.	Benson et al. 1996
Coriaria arborea *C. plumosa*	New Zealand	N. 16S rRNA gene partial sequences	Identical sequences for endophytes of both species indicating a separate lineage for these strains.	Benson et al. 1996
Discaria trinervis[a] *D. chacaye* *D. articulata*	Argentina	I. 16S rRNA gene partial sequence, RFLP of IGS 16S/23S rRNA genes, BOX-PCR	Local adaptation evidence at the host specific nodulation rate level although identity at 16S sequence level and RFLP analysis with diversity expressed in BOX-PCR fingerprint.	Chaia et al. 2006b
Coriaria arborea *C. plumosa*	New Zealand	N. 16S rRNA gene partial sequences	Two sequences differing only in one position, suggesting low diversity for the native *Coriaria* spp. as compared to exotic species in New Zealand.	Clawson et al. 1997
A.lnus glutinosa *A. cordata* *A. viridis* *Elaeagnus pungens*	New Zealand	N. 16S rRNA gene partial sequences	Exotic species in New Zealand harbour different strains that cluster separately with strain groups typical for each actinorhizal host.	Clawson et al. 1997
Casuarina equisetifolia	New Zealand	N. 16S rRNA gene partial sequences	Sequences may represent the original inoculated strain HFPCcI3.	Clawson et al. 1997
Elaeagnus angustifolia *Elaeagnus* sp. *Ceanothus americanus* *Colletia hystrix* *Talguenea quinquenervia* *Trevoa trinervis*	USA Chile USA Chile Chile Chile	N, I. 16S rRNA gene partial and full length sequences	Endophytes from Elaeagnaceae and Rhamnaceae form a distinct phylogenetic clade, except for those from *C. americanus* that cluster with strains infecting plants of the Rosaceae.	Clawson et al. 1998

Table 2 (continued)

Host plant	Location	Methods	Characterization of actinorhizal nodule endophytes or isolates.	Reference
Alnus incana *Myrica pensylvanica*	USA USA	N. 16S rRNA gene sequences	Dominance of one strain was evident in nodules collected from a single *M. pensylvanica* stand.	Clawson et al. 1999
Myrica gale *M. pensylvanica* *Comptonia peregrina*	USA Canada, Sweden, UK	N. 16S rRNA gene partial sequences	Variable strain diversity was found within Myricaceae: *M. pensylvanica* > *C. peregrina* > *M. gale*.	Clawson and Benson 1999
Chamaebatia foliolosa *Cercocarps ledifolius* 3 *Purshia* species	na[b]	N. 16S rRNA and *gln*A genes partial sequencing	Strains clustered with *Frankia* cluster I.	Clawson et al. 2004
Shepherdia canadensis *Alnus incana* *Myrica gale*	USA and UK	N, I. PCR-RFLP and complete sequencing of *rrs* gene	Distinct *Frankia* genotypes for each host species in sites of co-occurrence. *M. gale* had low strain diversity. *S. canadensis* strains belonged to a divergent subset of a cluster of Elaeagnaceae-infective strains and had high degree of diversity.	Huguet et al. 2001
Casuarina collina	New Caledonia	N, I. RFLP of 16S/23S IGS rRNA	Isolates fit into *Elaeagnus* infective Frankia, and belong to 4 ITS groups of *Gymnostoma* strains.	Gauthier et al. 1999
6 *Ceanothus* species	California	N. 16S rRNA gene partial sequence and rep-PCR	Occurrence of genetic diversity of *Frankia* in nodules. *Frankia* strains in nodules share a common ancestor to that of *Elaeagnus* infective strains.	Murry et al. 1997
Alnus nepalensis	India	N. PCR of ITS rrn operon, ARDRA, sequencing	*Frankia* community composition was strongly affected by altitude and to a lesser extent by site. In general soil properties of *A. nepalensis* rhizospheric soil did not correlate with *Frankia* genotypes.	Khan et al. 2007
6 *Gymnostoma* species	New Caledonia	N, I. 16S rDNA partial sequencing, nifDK IGS	*Gymnostoma* strains were close to one another, clustered with *Elaeagnus*-infective strains, and were distantly related to *Casuarina* and *Allocasuarina* strains.	Navarro et al. 1997
Casuarina equisetifolia *C. cunninghamiana* *Allocasuarina torulosa* 8 *Gymnostoma* species	Australia and New Caledonia	N, I. PCR-RFLP and sequencing of nif D-nifK IGS	*Casuarina* and *Allocasuarina* microsymbionts were in the same cluster, but those of *Gymnostoma* were closer to *Elaeagnaceae* strains.	Navarro et al. 1998

Table 2 (continued)

Host plant	Location	Methods	Characterization of actinorhizal nodule endophytes or isolates.	Reference
8 *Gymnostoma* species	New Caledonia	N. PCR-RFLP rrs-rrl (16S-23S) IGS	17 patterns among 358 strains, without strict specificity to any host plant. Pattern distribution was related to soil type and to host plant species.	Navarro et al. 1999
Casuarina equisetifolia	Mexico	I. 16S rRNA partial sequencing	Endophytes were unable to nodulate their host	Niner et al. 1996
11 *Ceanothus* species *Chamaebatia*	California	N, I. 16S and 23S rRNA gene sequencing	Low genetic diversity among *Frankia* strains nodulating *Ceanothus* at an elevational gradient or from a wider geographic range. Strains from *Chamaebatia* formed a single group with several *Ceanothus* symbionts.	Oakley et al. 2004
9 *Ceanothus* species	USA	N. 16S rRNA gene PCR-RFLP, and sequencing	4 *Frankia* groups that did not follow the taxonomic lines of *Ceanothus* host species. Strains were related to sample collection locale.	Ritchie and Myrold 1999
Casuarina equisetifolia *C. cunninghamiana* *Allocasuarina torulosa* *A. littoralis*	Australia	N, I. PCR-RFLP of *rrn* and *nif* regions	Higher diversity among uncultured strains than in those previously isolated.	Rouvier et al. 1996
Casuarina esquisetifolia	Mexico	I. 16S rRNA gene complete sequence, RFLP of rRNA gene and nifH sequence analysis.	Non-*Frankia* actinomycete N_2-fixing endophyte.	Valdés et al. 2005
Alnus acuminata	Argentina	I. PCR-BOX	High strain diversity that includes *Frankia* strains and non-*Frankia* actinomycetes.	Valdés La Hens 2007
4 *Rosaceae* species *Ceanothus* *Datisca glomerata*	California	N. 16S rRNA gene and glnA and ITS DNA sequencing	Low genetic diversity among *Frankia* strains that nodulate sympatric populations of actinorhizal plants in California, with no apparent host-specificity.	Vanden Heuvel et al. 2004

[a] *Discaria trinervis* has been renamed as *Ochetophila trinervis* (Kellermann et al. 2005).
[b] na: no information available.

3. Isolation of *Frankia*

3.1. Isolation from nodules

An exahustive review of procedures for the isolation from nodules and culture of *Frankia* strains can be found in Lechevalier and Lechevalier (1990). Crucial steps for isolation of the endophyte from nodules are the surface sterilization of root nodules and the isolation and growth media to be used. Strain isolation and growth may be favoured by adding special factors to the culture media, like the triterpene dipterocarpol purified from root lipid extracts of *Alnus glutinosa* (Quispel et al. 1983; 1989), or the flavonoid quercetin (Sayed and Wheeler 1999). Due to the slow growth rate of *Frankia,* contaminants are the major difficulty that arises during isolation trials. Enhanced growth and reduced doubling times in several *Frankia* strains were achieved by adding *Alnus glutinosa* seed extracts (Ringø et al. 1995) or phospholipids containing palmitoyl residues to the liquid growth media (Selim and Schwenke 1995). Recently a new solid growth medium was developed that allows colonies of *Frankia* HFPCcI3 to become visible with the unaided eye after an incubation period as short as three days (Bassi and Benson 2007). The medium contains gellan gum as a gelling agent and certain peptones. It would be interesting to find media with similar properties for isolation trials.

3.2 Isolation of *Frankia* from soil

Frankia as a soil organism can be found in different niches (Figure 2). Successful isolation of *Frankia* from soil is reported by Baker and O´Keefe (1984) who used soil from under *Cercocarpus, Ceanothus, Casuarina* and *Myrica*. Suspensions of soil in water containing phenol were separated by discontinuous sucrose gradient centrifugation and material from an interface yielded growth of *Frankia* in a minimal culture medium. Among the tested samples only sub-surface soils (20-30 cm depth) under *Cercocarpus montanus* gave rise to *Frankia* isolates whose N_2 fixation and infective capacities were unfortunately not evaluated by Baker and O´Keefe (1984). The lack of success with surface soils was thought to be due to its higher populations of contaminating organisms as these soils were richer in organic matter. It is interesting to note that *Cercocarpus* belongs to family Rosaceae, one of the families where *Frankia* has not yet been isolated from nodules.

There are probably many unsuccessful attempts to isolate *Frankia* directly from soil that have not been reported. In contrast to isolations from root nodules, isolation directly from soil eliminates the possible symbiotic selection by a host plant. However, also soil treatments and growth media can be selective. The increasing information about *Frankia* genomes and their corresponding enzymes are likely to be helpful to optimize media for isolation and growth of *Frankia* directly from soil.

An alternative attempt to isolate *Frankia* from soil is to use the soil as an inoculum of an actinorhizal trap plant and if nodules are formed, then the task is to isolate *Frankia* from those young tiny nodules (Figure 2 note 3), which sometimes are easy to get free of contaminants after nodule surface sterilization (Gtari et al. 2004; Maunuksela et al. 1999). It should be noted that most *Frankia* strains isolated from nodules do not originate from a single cell.

Bacteria are usually parts of a microbial community or consortium rather than occurring singly in soil (Figure 2, note 10). *Frankia* in soil is probably part of such a functional group, and these groups are supposed to be spatially organized in biofilm structures on soil particles, root surfaces or plant debris in the soil. We need to know more about saprophytic growth of *Frankia* in soil, as well as in interactions with other microorganisms - including biofilm formation by *Frankia*. Meanwhile, a functional genomic approach (Bertin et al. 2008) to soil microcosm studies of *Frankia* could be a valuable starting point.

4. Measurements of N_2 fixation by *Frankia* in soil

Unlike most rhizobia, *Frankia* can reduce N_2 with the aid of dinitrogenase both when cultured in N-free medium and when living in root nodules. So far, it is not possible to recognise *Frankia* as an N_2-fixing organism in soil (Figure 2 note 9), nor are we aware of any evidence for *Frankia* not being able to fix N_2 in soil. Measurements of N_2 fixation in soil requires sensitive analytical methods and are difficult to achieve by techniques other than acetylene reduction assays (ARA) where acetylene (ethyne) replaces N_2 as the substrate for dinitrogenase and results in the production of ethylene (ethene). Common difficulties with ARA in soil are that only small amounts of ethylene are produced from acetylene, and that ethylene can also be produced by non-N_2-fixing organisms such as fungi. This makes it difficult to determine if there is N_2 fixation in the soil. Additionally, when N_2 fixation is inferred from ARA it is not known whether the activity was due to *Frankia* and/or other bacteria in the studied soil.

As far as we know, quantitative-PCR (Real Time PCR) has not been used to detect *Frankia* in soil (see section 6). Instead of measuring dinitrogenase activity in soil, it might be possible to evaluate the specific expression of *Frankia nif* genes in soil, analysing environmental RNA with appropriate primers and q-PCR (VanGuilder et al. 2008).

5. Infection mechanisms and nodule development

Among actinorhizal symbioses, three of the eight host families (Myricaceae, Betulaceae and Casuarinaceae) are nodulated by *Frankia* via the intracellular infection pathway. In five of the families (Elaeagnaceae, Rhamnaceae, Rosaseae, Datiscaceae and Coriariaceae), early nodule initiation occurs, or probably occurs (only indirect evidence for the families Rosaceae, Datiscaceae and Coriariaceae), via intercellular colonization (Wall and Berry 2008). Mechanisms are briefly described as follows (Fig. 2).

In the intracellular infection, or root-hair infection pathway, *Frankia* induces deformation of the root hairs and in a deeply-folded region of these root hairs, *Frankia* filaments transit from the root-hair surface to the inner part of the root hair forming a structure within the root hair that is analogous to the infection thread found in legume-rhizobial symbioses. *Frankia* penetration of the root hair triggers cell divisions in the root cortex subadjacent to the infected root hair, forming a zone called the prenodule. Some of these newly-divided cells expand and subsequently become infected by *Frankia*. In *Casuarina glauca* it has been shown that the prenodule cells display the same differentiation as the corresponding nodule cells (Laplaze et al. 2000).

In the intercellular infection pathway, *Frankia* filaments invade the cortex of young roots by growing in the middle lamella between adjacent epidermal cells and cortical cells. Root hair deformation and cortical cell division to form prenodules are not induced. The host cells secrete extracellular material, creating an expanded intercellular zone.

Once *Frankia* has started the infection of the root, either intracellularly or intercellularly, a nodule lobe primordium is initiated in the root pericycle. As the nodule lobe primordium expands, the nodule cortex becomes infected intracellularly by *Frankia* filaments transiting through the infected area of the root cortex (either the prenodule or the intercellular infected tissue, depending on infection strategy) into the base of the primordium. Finally, the mature nodule develops from the apical meristem, *Frankia* vesicles differentiate within the cortical cells, and N_2 fixation is expressed.

6. Nodulation tests as a tool to study *Frankia* in soil

6.1 Qualitative tests

Studies of infective *Frankia* populations in soils are based on plant bioassays using selected host plants (Figure 2, note 4). Qualitative tests are simply done by planting seeds or non-nodulated plants into soil samples or soil in the field and then recording nodule appearance. This test is considered a basic one for *Frankia* because it is linked to the original definition of *Frankia* as the endophyte of actinorhizal nodules.

Considering the fact that nodulation is the result of using soil as inoculum, and that soil samples hold not only *Frankia* but the microbial community accompanying *Frankia* (see section 9 below), the positive result of nodulation should be confirmed by reisolation of *Frankia* from obtained nodules and/or by characterizing the endophyte to be *Frankia* by molecular methods. To some extent such confirmation has been done (Table 1).

6.2 Quantitative tests

One simple method for quantitative nodulation tests is to grow a large number of plants in a small soil volume and evaluate the frequency of nodulated plants and the number of nodules per plant (nodulation test, NT; Dawson et al. 1989; Zitzer and Dawson 1992). A more accurate quantification of infective *Frankia* in soils can be obtained by using serial dilutions of soil samples in plant bioassays. Such measures are expressed as nodulation units (NU) per g of soil or per cm^3 of soil (Figure 2 note 2).

The nodulation capacity method (NC) is based on the plant bioassays developed by Quispel (1954) and was refined by Van Dijk (1984). The abundance of potentially *Frankia* NU in soil samples may be determined from the relationship between the quantity of soil used as inoculum and the numbers of root nodules subsequently produced on test plants. The nodulation capacity of a *Frankia* source is defined as the number of *Frankia* particles per unit of soil, each of which induces one nodule in the nodulation test. By using dilution series of soils in nodulation tests with hydroponically grown test plants, the nodulation capacity can be calculated from the functional relationship between successive quantities of the soil

sample and the numbers of nodules produced. Calculations of the implicit nodulation capacity have been restricted to those data which show an approximately linear relationship between the quantity of inoculum and the nodulation level, *i.e.* at nodulation levels where the root biomass does not limit nodulation. A modification of this method, by fitting least-squares estimates to the linear function of nodule number versus inoculum quantity, allows for calculating means and variances (Paschke and Dawson 1993). A special case of nodulation capacity method was used by Wolters et al. (1997a) for waterlogged soils containing both effective and ineffective *Frankia* strains.

The most probable number (MPN) method is based on probability theory and relies on determination of the presence or absence of the organism of interest in several consecutive dilutions of the sample being tested. The pattern of nodulated and non-nodulated plants is then used to derive a population estimate, the MPN (Woomer et al. 1988). The NC method and the MPN method are thus based on different assumptions. When counting nodules in the NC method, the number of nodules is assumed to be directly proportional to the number of potentially-infective *Frankia*, at least up until all nodulation sites on the roots are saturated. Alternatively, the MPN approach is based on the assumption that potentially-infective *Frankia* are diluted to extinction and that a single infective unit will give rise to a nodule on a test plant. Estimates obtained using MPN and NC techniques were in agreement, especially between 30 and 300 NU g^{-1} soil, for *Alnus*-infective *Frankia* (Huss-Danell and Myrold 1994; Myrold and Huss-Danell 1994) (Table 1). Similar agreement was found between results obtained with MPN and the least square estimates method for *Myrica*-infective *Frankia* (Zimpfer et al. 1997) (Table 1). Numbers of NU by the MPN method were lower than by the NC method when calculated for *Ceanothus*-infective *Frankia* in soil under host plants, but this difference was not found in soils lacking host plants (Jeong and Myrold 2001) (Table 1). It has been suggested that the discrepancy between the two estimates could be due to low population levels in soil samples, as few as between 0.2 and 5.2 NU g^{-1} soil.

Neither the NC method nor the MPN method gives information about the numbers of early infection stages preceding nodule formation. For this reason, these methods describe nodulation capacity and not infective capacity.

In summary, reported numbers of NU per g or per cm^3 of soil vary considerably (Table 1). There is no single way to express the number of *Frankia* NU per unit of soil (Table 1). A possible standardized measure would be to express number of NU per g of dried soil (Huss-Danell and Myrold 1994; Van Dijk 1984). In spite of limitations described above, numbers of NU provide useful information about the soil and the possible role of *Frankia* in soil microbial communities. Numbers of NU may also give information about growth requirements for *Frankia* and thus help to design proper media for isolation and cultivation of *Frankia* from soil.

6.3 Factors influencing nodulation

6.3.1 Soil environment

The development of an actinorhizal root nodule results from the joint action of plant genotype, *Frankia* genotype, and the environment of the partners. A number of aspects on the soil environment and nodulation was recently discussed by Dawson (2008) and therefore we give only a brief summary here (Table 3). There are many abiotic and biotic factors acting together in the numerous sites and soils studied. Therefore, the abundant studies of hosts and *Frankia* genotypes provide valuable information also on the use of nodulated actinorhizal plants, such as in soil reclamation.

Quantitative estimates of NU in a soil require a lot of handling of soil samples. To obtain a homogeneous sample, and similar aliquots of the sample, the soil needs to be cleaned from stones, roots, litter, etc. and sieved. These steps together with suspending and shaking the soil samples will disturb the soil structure. The extent to which such disturbances affect the outcome of a nodulation test is still in question.

Various soil factors may affect nodulation when present in the samples to be tested for nodulation capacity (Table 3), and this is particularly difficult to assess at low dilution levels where soil inocula are found at higher concentrations. On the other hand, as nodulation tests support advantageous conditions for plant growth and nodulation, some soil factors are likely ameliorated in the tests and therefore results may not necessarily reflect the situation in field. Evidence of this was given by an irrigation experiment on *Ceanothus* seedlings growing in arid soils in Southern California chaparral where irrigated plants had a significant increase in nodulation frequency compared to those under natural conditions (Pratt et al. 1997). Discrepancies in nodulation between natural conditions and nodulation tests can also be due to

nutrient deficiencies in the soil environment that may be overcome by nutrient amendments under the experimental conditions. This was found in a baiting study with some soils from Australia which only caused nodulation when P amendments were performed (Reddell et al. 1986).

6.3.2 Plants

The use of dissimilar trap plant species can reveal differences in both number of NU and genetic diversity of *Frankia* in soil. A diversity of soil borne *Frankia* genotypes were found when plants of *Alnus, Myrica* and *Shepherdia* were tested with more than one hundred soil samples from a gradient of successional stages in a sand dune system at Lake Michigan. Nodular strains included one genotype for *Alnus* and three for *Myrica*, all of them belonging to a homogeneous cluster while in the case of *Shepherdia*, nodular strains were separated into two other genetically distinct clusters (Huguet et al. 2004; McCray Batzli et al. 2004). This is understandable as *Shepherdia* is in a different host infection group than *Alnus* and *Myrica* (Hahn 2008).

The use of different trap plant species within the same genus can yield different results in NU. This was demonstrated when three *Alnus* species were combined with two different nutrient solutions and two different ways of adding the soil inoculum (Huss-Danell and Myrold 1994). We collected forest soil from northern Sweden within the natural distribution of *Alnus incana*, and used *A. glutinosa* and *A. rubra* as additional test plants. The extent of nodulation was always in the order *Alnus rubra>A. incana>A. glutinosa,* irrespective of nutrient solution and how the soil inoculum was added. The difference between *A. rubra* and *A. glutinosa* ranged from 14 to 80 times when the species were compared within the same nutrient solution and addition of inoculum. Within the same plant species seed-lots from different localities may also affect nodulation by different strains. A variable degree of incompatibility to root nodule formation in *Alnus glutinosa* by ineffective *Frankia* strains was exhibited when seedlings obtained from seeds collected in different localities were inoculated with soils of wet dune slacks under *A. glutinosa* (Van Dijk and Sluimer-Stolk 1990; Wolters et al. 1999). Therefore, the choice of trap plant genotypes can be decisive when the aim is to determine number of NU in soils.

It is important to use test plants that are healthy, of similar size and grown under reproducible conditions. A large number of germinated seeds and good cultivation facilities should be available. In order to avoid contaminating *Frankia,* the common practice is to surface sterilize the seeds that will be used in nodulation tests. However, treating the seeds with oxidizing agents and large volumes of water can lead to a loss of compounds being positive or negative for infection by *Frankia*. For example, aqueous seed-washes of *Alnus rubra* and individual flavonoid-like compounds isolated from such preparations either enhanced or inhibited nodulation of *A. rubra* seedlings inoculated with *Frankia* strains (Benoit and Berry 1997).

6.3.3 Autoregulation

Autoregulation of nodulation in actinorhizal symbioses is a plant response that controls the final number of nodules, or nodule biomass, per plant. This is accomplished by a feed-back mechanism that inhibits further infection and nodule development after a threshold value of infections has been reached in the actinorhizal roots (Valverde and Wall 1999; Wall and Huss-Danell 1997). Autoregulation operates independently of *Frankia´*s infection pathway (Wall et al. 2003) and is turned on very early after inoculation, well before nodules are visible (Valverde and Wall 1999; Wall and Huss-Danell 1997). Although autoregulation was not known as a phenomenon when Van Dijk (1984) described the NC test, he noticed different factors affecting the evaluation of nodulation, such as inoculation period and nodulation period. He concluded that the NC method must be standardized, and he recommended use of the linear part of the nodulation curve at relatively high dilution levels. As a result, counting of nodules in an experiment should be done when the pre-requisites of the NC are fulfilled, and nodules should be counted up to the moment when the number of nodules does not increase. The NC method must be used cautiously due to autoregulation phenomena not known but envisaged at the time of Van Dijk´s work.

Table 3. *Frankia* nodulation and the soil environment

Details	References
Soil properties	
NC[a] of forest soils with *Betula* spp., but lacking actinorhizas had a positive correlation with soil pH.	Smolander and Sundman 1987
NC of *Frankia alni* populations in an acid forest soil increased after liming, but *Frankia* GU were not affected.	Hilger and Myrold 1992
Liming increased the NC of the humus layer of acid conifer forests.	Elo et al. 2000
Frankia ecotypic adaptation to soil moisture conditions seemed to follow the same trends as their host taxa.	McCray Batzli et al. 2004
High ground water tables at *Betula* sites produced the highest NC.	Van Dijk 1984
Irrigation of *Ceanothus* spp. after a wildfire in chaparral increased the nodulation frequency.	Pratt et al. 1997
NC of ineffective *Frankia* strain in wet dune slacks had a positive correlation to the period of soil inundation.	Van Dijk and Sluimer-Stolk 1990
Waterlogged *Alnus glutinosa* stands were *Frankia* infective, but part of the soils caused ineffective nodulation.	Wolters et al. 1997a
The nodulation of *Purshia* and *Cowania* in surface soils was positively correlated with precipitation.	Righetti et al. 1986
Moist and well aerated subsurficial alluvial sands had the highest NC in *Casuarina.*	Dawson et al. 1989
NC on *Discaria* of soils along a vegetation gradient increased under semi-arid conditions associated to watercourse.	Chaia et al. 2006a
Nodulation of Casuarinaceae was limited by low soil P status.	Reddell et al. 1986
The pioneer soils following deglaciation were *Frankia* infective only if supplemented with P.	Chapin III et al. 1994
NC on *Alnus* from forest soils with liming and/or NPK had a negative correlation with C which, rather than pH, would regulate *Frankia* infective populations.	Myrold and Huss-Danell 1994
NC on *Alnus* from red alder stands was most related to soil properties than to stand age. NU had negative correlations with NO_3^- and total C, and positive with pH.	Martin et al. 2003
The nodulation of *Myrica* in coastal soils from a barrier island was strongly affected by soil salinity and water.	Young et al. 1992
Spatial patterns of *Frankia* and *Myrica* in coastal soil were related to microtopography and soil chlorides.	Wijnholds and Young 2000
The nodulation of *Casuarina*, even at sites with high salt concentrations, suggests *Frankia* growth in the rhizosphere**.**	Reddell et al. 1986
Soil constraints to nodulation and N_2 fixation in arid and semi-arid environments. Review.	Reddell et al. 1991
Ecological factors influencing infective *Frankia* populations. Review.	Dawson 2008
Geographical and ecological patterns of actinorhizal symbionts. Review.	Benson and Dawson 2007
Frankia and soil environmental conditions. Review.	Valdés 2008
Distinct differences in NC between bottomland and upland were associated with soil order, pH, host presence and planted host seedlings.	Zitzer and Dawson 1992
Soil depth and altitude of sites	
Deeper soils from coastal sand dunes produced higher nodulation in *Hippophaë.*	Oremus 1980
Deeper soils from California caused an increased nodulation in *Purshia* and *Cowania.*	Righetti et al. 1986

Table 3 (continued)

Details	References
Myrica, Elaeagnus and *Alnus* infective *Frankia* were found throughout soil profiles to a maximum of 1.50 m depth.	Paschke et al. 1994
Soils devoid of Elaeagnaceae had declining NC in *Elaeagnus* with depth that was associated to lower soil organic matter. Strain diversity was maintained throughout the soil column, but the relative distribution of strains varied.	Nalin et al. 1997
NC of *Frankia* infective on *Casuarina* in Australia increased down to soil depths of about 60-80 cm.	Dawson et al. 1989
Distribution of *Frankia* genotypes in *Alnus* nodules were strongly affected by altitude, in Sikkim Himalayas.	Khan et al. 2007
Temperature	
Soil temperatures between 22 and 26ºC affected nodulation in *Ceanothus,* and inhibition was caused at 31ºC.	Wollum and Youngberg 1969
Season	
NC of soils in *Alnus* declined from spring to summer, although GU remained rather similar.	Myrold and Huss-Danell 1994
NC of Patagonian soils on *Discaria* varied seasonally and according soil water content.	Chaia et al. 2007
Soils with host plants	
Nodulation in *Casuarina* is common in regions where the genus is indigenous.	Bond 1957
Casuarina infective *Frankia* was detected adjacent, but not distantly, to the host trees (Jamaica).	Zimpfer et al. 1999
NC of *Frankia* infective on *Alnus* was affected by host plant presence.	Myrold and Huss-Danell 1994
Frankia population in soil from a *Ceanothus* stand was higher than in soil from a Douglas-fir stand.	Jeong and Myrold 2001
Soils without host plants	
Tropical wet and dry forest soils of Costa Rica lacking actinorhizas were infective on *Alnus* and *Elaeagnus*.	Paschke and Dawson 1992
Soil from savannah in Texas under *Condalia* (Rhamanceae) nodulated *Alnus.*	Zitzer et al. 1996
Reclaimed and agricultural soils devoid of actinorhizal plants nodulated native *Myrica* plants but not *Casuarina.*	Zimpfer et al. 1997
Discaria and *Colletia* were nodulated by rhizospheric soils of other rhamnaceous species growing at distant localities.	Cusato and Tortosa 1998
Soils under *Betula, Pinus* or *Picea,* devoid of actinorhizas nodulated *Alnus*. *Frankia* populations in nodules represented a fraction of infective *Frankia* in the soils**.**	Maunuksela et al. 1999
***Frankia* growth in the rhizosphere of non-host plants**	
Forest soils in Finland, with *Betula* but lacking actinorhizas had the highest infective capacity in *Alnus,* causing Sp- nodules.	Van Dijk 1984; Smolander 1990
Frankia strains were able to colonize and grow in the rhizosphere of *Betula, Poa* and *Festuca* seedlings without addition of a C source, causing growth increases in roots, shoots and/or root/shoot ratios in plants.	Rönkkö et al. 1993
Rhizospheric soils of *Alphitonia* (non-nodulated Rhamnaceae) caused higher nodulation in *Gymnostoma* than those from *Pinus* or bare soils.	Gauthier et al. 2000
NC of rhizospheric soil under *Alnus* and *Rubus spectabilis* was similar, and was much higher than under *Betula papyrifera*.	Markham and Chanway 1996

Table 3 (continued)

Details	References
Soils lacking *Frankia*	
Sandy soils, or alkaline clayey soil reclaimed from the sea were not infective in *Alnus.*	Houwers and Akkermans 1981
No indigenous Frankia infective on *Casuarina* was detected in soils from the Suez Canal University, Egypt.	Mansour and Baker 1994
Alnus infective *Frankia* was not detected in several circumpolar soils.	Huss-Danell et al. 1999
Allelochemicals	
NC of soil containing *Frankia* was increased with tissue extracts from *Casuarina* cladodes, but was decreased by the addition of organic binding agents.	Zimpfer et al. 2002
Soils with increasing levels of cladode concentration and an added *Frankia* isolate decreased in infectivity, while soils with native *Frankia* increased in infectivity on *Casuarina*.	Zimpfer et al. 2003
Strain competition	
NC of soil from adjacent Sp+ and Sp- nodules favour the Sp+ type by the release of more infective particles.	Van Dijk 1984
Competitive interactions between ineffective and effective *Frankia* strains seem to be restricted to long- term inundated alder vegetations.	Van Dijk and Sluimer-Stoll 1990
Introduced *Frankia* strains in a sandy loam from a natural stand of *Alnus* could compete for nodule formation with the indigenous *Frankia* population.	Zepp et al. 1997b
Interactions between *Frankia* and other soil organisms	
NC of soils inoculated with *Frankia* strain was the highest, suggesting a positive synergism between soil biota as a whole and *Frankia* inoculum with respect to host infection**.**	Zimpfer et al. 2003
Frankia NU and arbuscular mycorrhizal fungi infective units in *Discaria* were positively correlated in Patagonian soils.	Chaia et al. 2006a
Mycorrhizas in actinorhizal plants. Review.	Cervantes and Rodríguez-Barrueco 1992.
Successional stage	
Soil from sandy beach and from the early phase dunes produced low nodulation, but it increased in dunes of a later phase where *Hippophaë* had begun to invade.	Oremus 1980
Sp- nodules in *Alnus* occurred in young and old dune areas, while Sp+ nodules were mainly restricted to the old dune.	Van Dijk 1984
Oldest volcanic deposits, with higher soil moisture, organic matter and vegetation cover had highest infectivity in *Myrica*.	Burleigh and Dawson 1994a
The frequency of nodulated *Dryas* increased towards later portions of the sere in the primary succession within glacial forelands.	Kohls et al. 1994
Shepherdia-infective *Frankia* were more abundant in soils from drier and earlier successional sites, whereas *Alnus*- and *Myrica*-infective *Frankia* were more abundant in soils from later successional sites.	McCray Batzli et al. 2004

Table 3 (continued)

Details	References
Frankia host-specificity distribution and diversity in soils of a sand dune ecosystem rich in actinorhizal plant species varied with contrasting young and old seral plant communities.	Huguet et al. 2004
Soils under *Alnus* thickets, in a primary successional volcanic locale, had higher NC in *Alnus* than the other communities.	Seeds and Bishop 2009
Time without host plants	
Timber stands, devoid of *Ceanothus* up to 100 yrs, were infective but the older stands caused low nodulation in *Ceanothus.*	Wollum et al. 1968
Alnus introduced into a former agricultural land devoid of hosts at least 20 yrs were nodulated.	Weber 1986
Acid forest soils free of actinorhizal plants for 20 to more than 100 yrs nodulated *Alnus*.	Smolander and Sundman 1987
Dispersal	
Nodulation in *Casuarina* occurred only in places subjected to occasional flooding from near-by river or stream.	Bond 1976
The ability of *Frankia* to grow and sporulate outside nodules of *Casuarina*, inoculated with crushed nodules, would contribute to its dissemination in soil.	Diem et al. 1982
Irrigation would have favoured *Frankia* dispersal to plots devoid of *Alnus* in meadow and peatland soils.	Arveby and Huss-Danell 1988
Deposition of alluvial sediments could have supported the higher NC of a bottomland in a river flood plain.	Zitzer and Dawson 1992
Infective *Frankia* was attached to particles in river water, and could be carried by water movements to shores.	Huss-Danell et al. 1997
The occurrence of infective *Frankia* in lake sediments distant from lake shores supports the assumption of water dispersal.	Chaia et al. 2005
Elaeagnus infective *Frankia* was found on particles removed from air filters of a greenhouse.	Paschke 1993
There is evidence to suggest that *Frankia* may be dispersed by insects and other soil invertebrates.	Paschke 1993
Earthworms were capable of transmitting viable infective propagules of *Frankia* in the casts.	Reddell and Spain 1991
Bird nests, which did not contain soil as building material, contained *Alnus* and *Elaeagnus* infective *Frankia.*	Paschke and Dawson 1993
Frankia spores nodulated *Casuarina* after passage through the digestive tract of captive parakeets.	Burleigh and Dawson 1995
Soil reclamation	
Elaeagnus and *Shepherdia* inoculated with soil containing *Frankia* and mycorrhizal fungi, outplanted on oil sand tailings devoid of nutrients, had a superior nodulation than those uninoculated.	Visser et al. 1991
Frankia strains nodulated *Casuarina* plants irrigated with water outlets (containing cyanide, arsenic, mercury ions, and contaminating microorganisms) for further soil reclamation.	Sayed 2003
Chlorinated benzoates (contaminants in soil and water) had a negative effect on *Frankia* GU in microcosm systems. A similar reduction occurred in the NU of *Alnus* infective *Frankia*.	Ramirez-Saad 1999
Heavy metal mine tailings had a very low *Frankia* infectivity. Dual inoculation of *Alnus* with *Frankia* (from field nodules) and *Paxillus,* in pots with peat and mine tailings, favoured nodulation and plant survival.	Markham 2005

Table 3 (continued)

Details	References
Increasing Zn additions to *Discaria* grown in soil produced delayed nodulation and reduced number of nodules but not reduced nodule biomass per plant.	Cusato et al. 2007
Inoculated and uninoculated *Alnus* seedlings planted on placer mine spoil in a subalpine watershed were equally nodulated after the first growing season indicating the presence of infective *Frankia* in those soils.	Densmore 2005
Increased soil Cu concentration up to 100 ppm allowed nodulation on *Alnus* seedlings, but at higher Cu levels, plants nodulated only in limed soils.	Fessenden and Sutherland 1979
Urban polluted soils nodulated *Alnus*. Infective strains belonged to *Alnus* infecting cluster but strains from most contamined soils belonged to cluster normally associated with *Elaeagnus*.	Ridgway et al. 2004
Casuarina plantations for windbreaks and for wood and fuel production cover about 300,000 ha in a coastal area in the South of China.	Zhong et al. 2010
Discussion on the stimulation of rhizodegradation by soil microflora in the presence of alders and the use of actinorhizal and (or) mycorrhizal alders for rehabilitation of disturbed soils. Review.	Roy et al. 2007

[a] NC, nodulation capacity of *Frankia*

6.3.4 Dispersal and survival of *Frankia*

The dispersal of *Frankia* and the soil sampling are related aspects that should be considered when nodulation tests are used to study *Frankia* in the environment. Dispersal mechanisms (reviewed by Dawson 2008) may account for a heterogeneous distribution of *Frankia* in soil due to localized deposition of propagules as a consequence of nodule decay and mycelial growth, as well as water, wind and animal transport. Some examples of studies dealing with this subject are presented in Table 3 and are briefly discussed below.

Decay of nodule tissue plays an important role in the maintenance of *Frankia* population in soil (Van Dijk 1979). The rate of nodule decay would be similar in both Sp+ and Sp- *Alnus glutinosa* nodule populations as shown by nodulation tests at intervals during one year of incubation of nodule lobes in soil samples (Van Dijk 1984). The ability of *Frankia* to grow and sporulate outside the nodule probably contributes actively to its dissemination in soil. Extranodular mycelial growth, including hyphae, sporangia and spherical vesicles, has been observed in *Discaria trinervis* and *D. americana* inoculated with soils and in *Casuarina equisetifolia* seedlings growing in hydroponics inoculated with a suspension of crushed nodules (Cusato and Tortosa 1990; Diem et al. 1982).

Water may be considered a dispersal agent for a short distance, especially from the bulk soil to the rhizoplane. It is not likely to account for long distance transport except in riparian environments (Paschke and Dawson 1993). Outside irrigated plots where nodulated *Alnus incana* were introduced, the horizontal spread of *Frankia* in peat apparently depended on water movements (Arveby and Huss-Danell 1988). Bond (1976) noted that nodulation in *Casuarina cristata* growing in Australia appeared to occur only where the trees were subject to occasional floods from nearby rivers or streams. Infective *Frankia* were found attached to heavy particles (from less than 0.0014 to up 0.066 mm) in river water, which could be carried by water movements to shores (Huss-Danell et al. 1997). Furthermore, the occurrence of infective *Frankia* in lake sediments supports the hypothesis of water dispersal (Chaia et al. 2005; Huss-Danell et al. 1997). Water movements would allow *Frankia* propagules to be removed from superficial sediments allowing dispersion to other shores. Deposition of alluvial sediments could probably support the higher nodulation capacity in *Alnus glutinosa* and *Elaeagnus angustifolia* of a bottomland located in a river flood plain (Zitzer and Dawson 1992). In contrast to spreading by water, there are few reports on wind dispersal of *Frankia*. One example of wind dispersal is from the presence of *Elaeagnus* infective *Frankia* on particles removed from greenhouse air filters used for 12 months (Paschke 1993).

Birds have been active in transport of infective *Frankia* propagules (Burleigh and Dawson 1995; Paschke and Dawson 1993). Findings include those of infective *Frankia* in bird nests which did not contain soil as building material, and of a *Frankia* strain nodulating *Casuarina equisetifolia* after passage through the digestive tract of captive parakeets (*Melopsittacus undulatus).* Additional evidence on vertebrate animals as dispersal agents was given by the finding of *Discaria trinervis*-infective *Frankia* in field collected faeces of cow, horse, sheep, wild boar and deer (M Sosa, E Raffaele and E Chaia, unpublished results). Moreover, viable infective propagules of *Frankia* may be transmitted by invertebrates like earthworms (Reddell and Spain 1991).

In some soils the role of dispersal appears limited. The *Frankia* gentoype composition of forest floor and canopy soils on the same *Alnus rubra* tree was not always identical, suggesting that canopy root nodulation was not restricted by dispersal but rather by environmental conditions in small spatial areas (Kennedy et al. 2010). Representative sampling of soil then becomes crucial.

The result of a nodulation test is dependent on soil storage conditions. Different procedures have been employed to estimate number of NU with respect to soil conditioning after sampling. Soils were used soon after sampling (Elo et al. 2000; Markham and Chanway 1996) or kept for several weeks or months (Paschke et al. 1994; Zimpfer et al. 1997), stored moist in cold conditions (Huss-Danell and Myrold 1994; Zitzer and Dawson 1992) or air-dried (McCray Batzli et al. 2004; Zimpfer et al. 1997, 1999) (Table 1).

Temperature of the soil samples is also important (Sayed et al. 1997). It is common to store soil samples at cold temperature but freezing of soils and subsequent storage at -20 ºC for 4 years had a large negative impact (Maunuksela et al. 2000). The nodulation capacity decreased to about 2 to 7% of the nodulation capacity of fresh soils. Moreover, analysis of *Frankia* in the nodules of *Alnus incana* trap plants revealed shifts in nodule forming *Frankia* populations (Maunuksela et al. 2000); however, it was not clear that the effects were due to freezing/thawing, storage time or both.

Superficial soils are commonly exposed to dry periods in the field; therefore, air-drying of soil samples followed by dry storage should be a gentle and natural treatment (Burleigh and Dawson 1994a; Chaia et al. 2007; Tortosa and Cusato 1991; Zimpfer et al. 1997). When soils were air-dried for 1 to 2 weeks their nodulation capacity decreased by 50-90 % as studied on *Alnus* (McCray Batzli et al. 2004) or *Discaria trinervis* (Chaia et al 2007). After that the air-dry soils had similar nodulation capacity on *D. trinervis* when stored for 1 week, 6 months or 12 years (Chaia et al. 2007). Lyophilization did not affect the nodulation capacity of these soils as compared to soils stored air-dried (Chaia et al. 2005).

Several mechanisms for *Frankia* survival in soils have been proposed. Strains exposed to drying can survive in deeper soils (Dawson et al., 1989), can tolerate desiccation by means of spores, or may increase trehalose concentration in hyphae (Burleigh and Dawson 1994b). Not only dry conditions can permit a long retention time for nodulation capacity of *Frankia* in nature. Strains from 30 years old lake sediments were able to nodulate *Alnus incana* (Huss-Danell et al. 1997), and strains from up to 50 years old lake sediments nodulated *Discaria trinervis* (Chaia et al. 2005).

6.3.5 Plant and *Frankia* growth conditions

Quantitative estimates of NU in a soil require a stepwise dilution of the soil and the use of the dilution steps as inoculum. When cultivating trap plants in solid substrates, the soil can be mixed in a series of proportions with a sterile inert solid substrate such as sand, perlite, vermiculite or glass beads. Solid substrates have the advantage of being natural substrates for plants as they provide physical contact for roots. In addition, aeration and moisture can resemble a soil. One disadvantage is that examination of plants for nodules can not be made without disturbance to the plants. In perlite roots tend to grow into perlite grains.

For a rapid and repeated examination of root systems, trap plants may be grown in liquid culture or in 'growth pouches' (Mega International, Minneapolis, USA). Since test plants are small, they do not consume large amounts of nutrients and the nutrient solutions should therefore be dilute. Nitrogen is well-known to inhibit nodulation (*e.g.* Gentili and Huss-Danell 2002; 2003; Gentili et al. 2006; Hiltner 1895; Huss-Danell 1997; Wall et al. 2000) and should be kept at low concentration. Phosphate, on the other hand, has been shown to have a positive effect on nodulation, at least in *Alnus glutinosa, A. incana*, *Hippophaë rhamnoides* and *Discaria trinervis* (Gentili and Huss-Danell 2002; 2003; Gentili et al. 2006; Quispel 1958; Valverde et al. 2002; Wall et al. 2000). A disadvantage of liquid cultures is that the roots do not have a natural physical environment and the nutrient solution has to be renewed repeatedly during the experiments. Either the inoculated seedlings have to be moved to new vessels with a new nutrient solution, or only the solution can be renewed in the same vessel. In both cases, the bulk of the inoculum is removed, and different results on nodulation are achieved (Huss-Danell and Myrold 1994; Van Dijk 1984). The removal of inoculum is a different situation than the use of a solid substrate where roots, due to growth, may reach increasingly more nodulating units during the experiment. It also may be that roots stimulate the growth of *Frankia* and therefore increase the number of NU in the test soil.

Growth pouches are intermediate to solid substrates and liquid cultures as they provide some surfaces to which the roots can attach. A disadvantage of pouches is the need for frequent attendance to keep moisture at a reasonably stable level. A great advantage of growth pouches is however that position of root tips and nodules can be marked on the pouch and time course and localisation of nodule development can be closely observed (Gabbarini and Wall 2008; Valverde and Wall 1999; Wall and Huss Danell 1997). Light, especially far-red light, has been reported to inhibit nodulation in legumes (Lie, 1974) and, although this effect has not been confirmed in all nodulated plants, the cultivation system should keep the roots darkened.

It is essential to include negative controls in all cultivation systems (such as when no soil is added) in order to decide that nodules are caused by the studied soil and not by contaminations. Including positive controls is also important and test plants supplied with a known source of *Frankia* added to the soil should be tested. If positive controls do not show nodules, it is very likely that the soil to be tested and/or the environmental conditions during the bioassay were somehow inhibiting nodulation. In such cases, any soil samples that do not cause nodulation cannot be evaluated properly.

The length of time that nodulation tests should be run is an interesting question. If no nodules appear, it may seem logical to wait several months. But, why does it take so long to obtain nodules? Is

it because of unhealthy plants or suboptimal conditions for growing the plants? Perhaps there were few nodulating *Frankia* units in the test soil, but with time they have multiplied into high enough numbers to be detected as NU? Or, maybe the test soil was lacking nodulating *Frankia*. Positive controls would help to distinguish between possible causes when no nodules appear. Still, when the test soil results in nodulated plants the duration of a nodulation test can be questioned. When nodules start to appear, it is likely that increasingly more will be formed as the plants are growing and forming new root tips. When numbers of NU are estimated according to the nodulation capacity method, it is important to count the number of nodules per plant and duration of nodulation tests may therefore affect the result. According to the MPN method, plants are simply scored as being nodulated or not. However, after enough time the few *Frankia* units in a dilution step of the soil may multiply into high enough numbers to result in nodules (Huss-Danell and Myrold 1994).

Table 1 summarizes studies dealing with the presence of *Frankia* in soils, both with and without host plants. Information about study methods and about strains characterization from nodules or soils are included. Although the outcome of a nodulation test depends on many factors, it is likely that nodulation tests will roughly describe an actual situation where some soils are richer than others in terms of nodulating *Frankia*.

7. Studies of *Frankia* in soil by DNA methods

A range of molecular methods have been applied to studies of *Frankia* in soil (Hahn et al. 1999). Most studies are based on environmental DNA samples analysed by PCR techniques. These require the design of specific primers or alternatively specific probes that have been used for *Frankia* detection in nodules (Baker and Mullin 1994; Hahn et al. 1997; Maunuksela et al. 1999) or directly in soil (Hahn et al. 1999; Mirza 2007; 2009).

In principle, two strategies have been developed to recover DNA from soils. One strategy is to separate microorganisms from other soil components. The bacterial suspension is subsequently treated to extract DNA or RNA according to established protocols. However, soil DNA extraction methods that rely on cell extraction prior to lysis are inefficient for filamentous organisms such as *Frankia* (Hilger and Myrold 1991). The second strategy involves *in situ* extraction of nucleic acids from microorganisms, the lysis being conducted directly on environmental samples. Direct lysis with detergents and physical cell wall disruption increases yield; nevertheless, the DNA is still contaminated with humic substances. Several protocols for direct lysis followed by purification of the DNA were developed (*e.g.* Hilger and Myrold 1991; Jeong and Myrold 2001; Myrold et al. 1990; Myrold and Huss-Danell 1994; Picard et al. 1992) and continue to be improved. Table 2 presents examples of studies representing the diversity of *Frankia* in soils provided by different methodologies and *Frankia* sources.

7.1 Quantitative studies

The first attempt to quantify *Frankia* in soil was to use rRNA directly extracted from soil for detection with oligonucleotide probes (Hahn et al. 1990). Further quantitative measures, expressed as genomic units (GU) per g or per cm^3 of soil, used PCR reactions applied to DNA extracts after a dilution series according to MPN techniques (Myrold and Huss-Danell 1994; Picard et al. 1992) (Figure 2 note 1). Alternatively, quantitative PCR could be used to measure environmental DNA with specific *Frankia* target DNA sequences. At this time we are not aware of any attempt to apply quantitative PCR technique to *Frankia* in soil samples (pers. comm. by several *Frankia* researchers).

Whether molecular studies are qualitative or quantitative, it is essential to have primers specific for *Frankia*. In early studies this was a limiting factor; however, more sequence information from *Frankia* is now available and various primers targeting different genes have been used (reviewed by Hahn et al. 1999; Hahn 2008). Today complete genomes are sequenced from three *Frankia* strains (Normand et al. 2007a). New information about more genomes is underway for comparative genome analysis and functional genomic studies (Alloisio et al 2010; L Tisa and P Normand; unpublished). Thus, more specific primers for PCR of *Frankia* DNA can be designed and used to study *Frankia* in soil. Nevertheless, all the molecular methods based on the amplification of a single gene have the limitation of mismatch, including the best specific primer sequences and the use of different genes. Underestimation in the case of gene multiple copies in a single organism, and overestimation of the

data, can occur because of the possibility of horizontal gene transfer between bacteria from different species in soil (Gogarten and Townsend 2005).

7.2 Genetic diversity

Molecular studies are useful not only to obtain the figures of GUs but also to reveal the genetic diversity of *Frankia*. While genetic information on *Frankia* in soil is limited, studies on *Frankia* in root nodules from field has provided information on the identity and diversity of some of the infective *Frankia* in the soil surrounding the nodules (reviewed by Hahn 2008) (Table 2). Molecular analysis of *Frankia* diversity in different soils under non-actinorhizal plants, combined with the characterization of *Frankia* captured from those soils into nodules of trap plants, suggested that different fractions of *Frankia* diversity are activated for nodulation under the influence of different non-host plants (Maunuksela et al. 1999).

Molecular analysis of *Frankia* isolates from nodules of different actinorhizal plants such as *Alnus* spp. and *Myrica* spp. inoculated with the same soil, and the characterization of *Frankia* soil population on the basis of 16s rDNA gene, suggest that in some cases *Frankia* populations in soils are dominated by a single strain or a group of closely related strains of *Frankia* (Clawson et al. 1999). Molecular tools combining PCR techniques and different genes will allow specific *Frankia* primers to be designed for studies of *Frankia,* especially in those cases of actinorhizal plants where isolates are not yet available (Table 2).

8. Comparisons of nodulation tests and DNA studies in soil

A qualitative comparison between *Elaeagnus*-infective *Frankia* and soil-borne *Frankia* was performed by Nalin et al. (1999). DNA samples extracted from pairs of nodules and surrounding soil at three soil depths were found to be congruent in terms of genetic polymorphism, as based on DNA analysis by specific 16S rDNA-targeted probes, partial PCR amplified sequences of 16S, and RFLP of *nif*D-K IGS.

Quantitative comparisons have also been tried (Table 4). Nodulation tests that estimate number of NU, and quantitative molecular DNA analyses that estimate number of GU (Figure 2, notes 1, 2), have been applied to the same soil samples (Myrold and Huss-Danell 1994). In all cases the number of NU was only a very small fraction, such as a few percent of the corresponding number of GU (Table 4).

There are several possible reasons for a low NU:GU ratio. Numbers of GU might be overestimated if the primers used in PCR reactions were unspecific and caused detection of microbes other than *Frankia*. Numbers of NU might be underestimated if the performance of nodulation tests did not allow all NU to be detected. But, it may be that the low NU:GU ratios are true values that simply express the ratio of nodulating *Frankia* out of total *Frankia*. We can assume that in a *Frankia* mycelium each cell is detected as one GU, but only a few cells in the mycelium are actually infective units giving rise to nodules. Consequently, determination of NU:GU ratios can give information about physiology of *Frankia* in soil. Supporting this hypothesis, NU:GU ratios tended to be higher when soils were sampled early in summer (June) than when soils from the same experimental plots were sampled in autumn (September). Meanwhile, numbers of GU stayed fairly similar. It is possible that soil conditions may have caused *Frankia* strain(s) to be infective in early growing season, perhaps because of a flush of nutrients at this time (Myrold and Huss-Danell 1994).

Other unknown reasons could be operating in the physiology of *Frankia* in soils. The effect of liming and presence of rhizospheres on natural *Frankia* populations in a forest soil (pH 4.7) was evaluated by *Alnus rubra* nodulation test and quantification of *Frankia* DNA in soil. After three months, numbers of NU in limed soils (pH 5.7) increased independent of which plants were growing nearby, while the number of *Frankia* GU was not affected. The change in *Frankia* physiology was expressed by the proportion of the total soil *Frankia* population that was infective and the 16-fold increase after liming (Hilger and Myrold 1992). So far NU:GU ratios appear to be determined only for *Frankia* that are infective on *Alnus.* It would be interesting to compare data from other actinorhizal plants from a variety of soils and seasons.

Table 4. *Frankia* populations in soils measured by nodulation tests and PCR- MPN techniques. (Based on Myrold and Huss-Danell 1994, Myrold et al. 1994). -, no data available.

Location	Nodulation tests (NU g^{-1}soil)	PCR-MPN (GU g^{-1} soil)	NU / GU (%)	DNA extracted mg g^{-1} soil
Finland	0.2 – 2940	-	-	-
Washington, USA	62	10000	0.6	5.1
Sweden (June)	50 – 182	1700 – 3400	4.6	11.4 – 20.8
Sweden (September)	25 – 60		1.7	8.5 – 27.2
Oregon, USA	593	92000	0.6	9.5
France	-	20000	-	50

9. Interactions between *Frankia* and other soil microorganisms

9.1 Bacteria

Frankia in soil is interacting with its host plant species in the presence of other microbes, even though it is possible to obtain functional nodules in laboratory conditions by inoculating axenic actinorhizal host plants with an appropriate *Frankia* culture. There are several experimental facts which suggest that *Frankia* participates in such multipart interactions during infection of the host roots and nodule induction (Figure 2, note 6). First, some non-N_2-fixing (atypical, Fix^-) *Frankia* isolates have been obtained from actinorhizal nodules (Baker et al., 1980; Hahn et al., 1988; Ramirez-Saad et al. 1988; Mirza et al. 1992). Additionally, non-*Frankia* N_2-fixing actinomycetes have been isolated together with typical *Frankia* strains from nodules of *Casuarina equisetifolia* (Valdés et al. 2005), *Coriaria myrtifolia* (Trujillo et al. 2006), *Elaeagnus angustifolia* (Gtari et al. 2004; 2007a) and *Alnus acuminata* (Valdés La Hens 2007). It is worth noting that in none of these cases of non-*Frankia* isolations has it been reported that periderm of field nodules has been removed prior to surface sterilization and isolation. Thus we can not assure that those non-*Frankia* N_2-fixing actinomycetes are true endophytes or just saprophytic microbes living on the surface or in periderm of the nodule.

Frankia interaction with host plants can involve several bacteria. Root nodule formation has been observed upon re-inoculation with Fix^- *Frankia* (Hahn et al. 1988; Van Dijk and Sluimer 1994) as well as non-*Frankia* N_2-fixing actinomycetes (Valdés La Hens, 2007); isolated strains from surface sterilized nodules of *Alnus glutinosa*, *Casuarina glauca* and *Elaeagnus angustifolia* had the ability to produce indole acetic acid, cellulase, chitinase and antagonism activities (Ghodhbane-Gtari et al. 2010); some bacteria like *Pseudomonas cepacia* can enhance nodulation by *Frankia* inoculated on *Alnus rubra* seedlings, and were considered as 'helper bacteria' in the nodulation process (Knowlton et al. 1980; Knowlton and Dawson 1983); different strains of rhizospheric actinomycetes belonging to the genera *Streptomyces, Actinoplanes and Micromonospora*, isolated from the rhizosphere of *Discaria trinervis* plants, promoted nodulation and consequently plant growth in *Discaria trinervis* when co-inoculated with *Frankia* (Solans 2007); the plant growth promotion of nodulated *Alnus glutinosa* seedlings, after inoculation with *Pseudomonas* and *Bacillus* strains isolated from the rhizosphere of the same host further suggests a multipart interaction regarding nodulation, symbiosis and plant growth. All together these independent observations suggest that *Frankia* interaction with its host plant is more complex than just an interaction between only two partners.

The coexistence of interacting microbes in the rhizosphere implies a complex network of signal exchange which modifies the physiology of the different partners in the interaction leading to infection, nodule development, nodule function and plant growth (Probanza et al. 1996). Bacterially produced phytohormones might be key signals for root growth and nodulation (Probanza et al. 1997; Solans 2007).

Comparative analysis of the complete genome of three *Frankia* strains belonging to different cross inoculation groups, ACN14a (*Alnus*), CcI3 (*Casuarina*) and EAN1pec (*Elaeagnus*), suggests that *Frankia* has low plant cell wall degrading capacity (Mastronunzio et al. 2008). This observation calls for attention to the role and mechanisms by which helper bacteria stimulate infection and nodulation by *Frankia*. For instance, rhizospheric actinomycetes isolated from field roots and nodules of *Discaria trinervis* were selected on the basis of their activity to degrade plant cell wall components such as cellulose, hemicellulose, pectin and lignin (Solans and Vobis, 2003), and those isolates behaved as helper bacteria which stimulate nodulation of *Discaria trinervis* by *Frankia* (Solans 2007). It is not known to what extent such interactions between helper bacteria and *Frankia* may be weakened when dilute soil suspensions are used in nodulation assays. In addition, the nodules obtained from soil samples in nodulation assays have usually not been investigated further with respect to characteristics or identitiy of *Frankia* or other bacteria occurring as endophytes in those nodules.

9.2 Mycorrhiza

All actinorhizal plant species examined in the field have been found to be mycorrhizal (Cervantes and Rodríguez-Barrueco 1992). Some actinorhizal species can be infected with both arbuscular mycorrhizae and ectomycorrhizal fungi simultaneously, forming a tetrapartite symbiosis, like in *Alnus* spp. (Becerra et al. 2005a; 2005b; Chatarpaul et al. 1989; Cruz-Cisneros and Valdés 1990), *Ceanothus coeruleus* and *Coriaria ruscifolia* (Cruz-Cisneros and Valdés 1990). Others can have one of the symbiotic fungi (tripartite symbiosis), like *Discaria* spp. and *Colletia hystrix* (Fontenla et al. 1998; 2001), and *Myrica cerifera* (Semones and Young 1995). Mycorrhizas increase the plants access to mineral nutrients, particularly nutrients with poor mobility or those present in low concentration in soil, like phosphate, ammonium, zinc and copper. Thereby the root environment is modified which, in turn, affects microbial populations in the rhizosphere (Barea et al. 2005).

The increased nodulation in *Discaria trinervis* inoculated with soils, as well as *Alnus* spp. inoculated with *Frankia* and arbuscular mycorrhizal inocula, pointed at a positive interaction between actinorhizal and mycorrhizal symbioses (Chaia et al. 2006a; Fraga-Beddiar and Le Tacon 1990; Russo 1989), where a synergistic effect would contribute to a modulated regulation of nodulation by the plant (Fraga-Beddiar and Le Tacon 1990). A common pathway of plant-microbe interaction that is part of the nodule developmental programme in actinorhizal plants has recently been demonstrated for legumes, arbuscular mycorrhiza and actinorhizal symbioses (Gherbi et al. 2008). Although there is a common pathway between nodulation and mycorrhiza formation, a detailed analysis of nodulation in the presence or absence of arbuscular mycorrhizal infection using localized inoculation of both symbionts suggested that there is no interference between nodulation and mycorrhiza formation (Obertello 2001). This corroborates the co-operative but not competitive interaction between *Frankia* and *Glomus* for nodulation and mycorrhizal colonization earlier found in *Casuarina equisetifolia* (Sempavalan et al. 1995).

10. Concluding remarks

Frankia in soil (Figure 2) can be thought to occupy different niches that are not necessarily linked to the corresponding actinorhizal host plant. Methods to isolate *Frankia* directly from soil need to be developed. Until then, nodules will continue to be the only source for isolation of *Frankia* strains. Current knowledge about *Frankia* in soil is based on complementary approaches: genetic analyses of nodules from the field, nodulation assays and DNA studies of soils (Tables 1 and 2). Nodulation tests are needed to describe presence and quantity (NU) of *Frankia* being infective on the different host species, but the information obtained is limited to infective *Frankia* populations for the corresponding plant species used in the nodulation test. Standardization of experimental procedures to evaluate nodulation capacity of soils is recommended for rational comparisons of different studies. Several reasons have surfaced. There are strong effects on plant nodulation exerted by experimental procedures such as soil handling before inoculation, *Frankia* strains and the environment of plant. There is also the occurrence of phenomena that depend on each symbiotic pair (such as specific recognition and autoregulation), and the relationships and effects of other soil microorganisms

introduced when soil inocula are used for formation of actinorhizal symbioses. DNA based methods can be applied to give both a measure of total *Frankia* populations (GU) in soil and to describe genetic diversity of the *Frankia* population in field nodules regardless of previous isolation of *Frankia*. DNA based methods are more powerful than nodulation tests in terms of revealing *Frankia* populations because they could be directly applied, by-passing plant trap assay, in all niches where *Frankia* is suspected to occur. When numbers of NU and GU have been compared, so far only for *Alnus*-infective *Frankia*, the number of NU has amounted to only a few percent of the number of GU. Such comparisons can give valuable information about physiology of *Frankia* in soil and a help to understand the role of *Frankia* in soil microbial communities. In terms of methods, nodulation tests can be improved by careful performance and standardised methods and DNA methods are likely to benefit from improved knowledge about *Frankia* genomes when designing primers for PCR reactions. As a soil organism *Frankia* is part of a microbial community with a complex network of signals and recognition where most probably both cooperations via physiological complementation between organisms and competition for resources occur. This point may explain difficulties to isolate *Frankia* directly from soil when trying to cultivate it out of its natural consortium. The unknown physiology of *Frankia* in soil, albeit its presence has been worldwide proved, has implications regarding the limitations of the definition of the genus *Frankia* apart from its symbiotic interaction with actinorhizal plants. Definitely, there is still a lot to study in this field, in the field.

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This paper is dedicated to the memory of Antoon D L Akkermans (1940-2006) for his pioneering and always creative and inspiring work on *Frankia* and actinorhizal plants, ranging from ecosystems worldwide to physiology and molecular biology of *Frankia* and its symbioses.

12. References

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