INEFFECTIVE *FRANKIA* IN WET ALDER SOILS

Rijksuniversiteit Groningen

INEFFECTIVE *FRANKIA* IN WET ALDER SOILS

Proefschrift

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van
de Rector Magnificus, Dr. D. F. J. Bosscher
in het openbaar te verdedigen op
vrijdag 18 december 1998
om 14.45 uur precies

door

Diederick Johannes Wolters
geboren op 2 december 1967
te Assen

Promotor: Prof. Dr. Ir. J. W. Woldendorp

Referents: Dr. A. D. L. Akkermans

Dr. C. van Dijk

Manuscriptcommissie:	Prof. Dr. L. J. Forney
	Prof. Dr. P. J. C. Kuiper
	Prof. Dr. J. A. van Veen
Paranimfen:	Dr. A. van Hinsberg
	H. C. Ramírez Saad

The studies described in this thesis were carried out at the Centre for Terrestrial Ecology of the Netherlands Institute of Ecology in Heteren, and at the Laboratory of Microbiology, Department of Biomolecular Sciences of the Wageningen Agricultural University in Wageningen, The Netherlands.

Ontwerp omslag: Albert Rademaker BNO, Annen
Druk: Ponsen & Looijen BV, Wageningen

CONTENTS

Chapter 1

GENERAL INTRODUCTION

NITROGEN-FIXING SYMBIOSES

In most terrestrial plant-dominated ecosystems, the limiting nutrient factors for plant growth are nitrogen (N), phosphorous (P), or potassium (K). While green plants are able to obtain carbon by means of photosynthesis, N (and P) has to be taken up from the soil in the form of ammonium (NH_4^+), and nitrate (NO_3^-) ions. These nitrogen compounds are recycled by micro-organisms from organic compounds, and in most cases the nitrogen-cycle in soil functions sufficiently to support plant growth.

In some ecosystems the availability of nitrogen is limited. This may be the case in primary soils (dune areas, sites of recent volcanic activity), but may also be caused by erosion of the topsoil by flash floods or landslides, acidic or oxygen-deprived conditions (reduced nitrogen mineralisation), and human exploitation (e.g. deforestation, crop harvesting, open mining). Several free-living bacteria of different genera (i.e. *Azotobacter*, *Bacillus*, *Clostridium*, *Klebsiella*, and cyanobacteria (i.e. *Nostoc*, *Anabaena*) have the ability to reduce atmospheric dinitrogen gas (N_2) to ammonium. The impact of these free-living nitrogen-fixers on the total soil nitrogen in general is modest, due to shortage of energy, but in the long run they contribute to the nitrogen enrichment of the soil. Alternatively, N_2-fixing bacteria that live in symbiosis with higher plants have better opportunities to grow due to the direct C-supply by their hosts (Ishizuka 1992). These symbiotic associations consist of specific nitrogen-fixing bacteria and (higher) plants, which contain the bacteria inside specialised tissues, mostly in or near the root system.

Phylogeny of actinorhizal plants

Whereas the symbioses of rhizobia (i.e. *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*) are confined to the Leguminosae and to the far-related genus *Parasponia* (Akkermans and Van Dijk 1981), the actinomycete *Frankia* is associated with a large number of higher plant genera. The majority of the participating "actinorhizal" plant species (so far about 200 species, belonging to 25 genera within 8 plant families) are woody perennials, and can be found in most climatic zones, usually as pioneer species early in vegetation successions (Bond 1983). In Europe and Asia the most important genera are *Alnus*, *Hippophaë*, *Myrica*, and *Elaeagnus*. The most common genus in tropical- and subtropical regions (Asia and Australia) is *Casuarina*. In arctic zones *Alnus* and *Dryas* are important genera, involved in colonisation of glacial wastelands (Lawrence *et al.* 1967, Ugolini 1968).

Initially, the taxonomy of the host plants of *Frankia*, based on classic plant morphology and anatomy data, was thought to be too diverse to be explained by a common descent. Bond (1983) therefore suggested that these associations arose individually, at a time when available soil nitrogen was universally scarce, thus providing the ancestors of present-day host plants with an additional nitrogen source. A recent analysis of the taxonomy of flowering plants, using the chloroplast gene *rbc*L, placed plant families with nodular nitrogen-fixing symbioses, both actinorhizal or rhizobial, together in a single clade, "rosid I" (Chase *et al.* 1993, Mullin and Dobritsa 1996). A study on a more narrow selection of plant species provided some evidence for the monophyly of this nitrogen fixing clade (Soltis *et al.* 1995). Recently, Swensen (1996) combined molecular genetic data with morphological characteristics to show that within this clade actinorhizal plant taxa form four major groups. Each of these groups is separated from the other lineages by related, but nonactinorhizal taxa, and also within each group, non-actinorhizal plant taxa were present. From the combined molecular and morphological data, Roy and Bousquet (1996), Swensen (1996), and Swensen and Mullin (1997) concluded that nodule-based nitrogen-fixing actinorhizal symbioses may have originated independently at least four times (Fig. 1). This indicates that this entire group of plant taxa (including the Leguminosae) has (or had) the inherent capacity for symbiotic relationships with soil microorganisms.

The phylogeny of some actinorhizal plant genera has previously been studied through RFLP analysis of rDNA of several *Alnus* spp. (Bousquet *et al.* 1989). This research has confirmed the taxonomy of alders based on morphological characters. The *Alnus* species clustered within the two subgenera Alnobetula (*A. crispa* and *A. viridis*) and Alnus (*A. incana*, *A. rugosa*, *A. japonica*, *A. glutinosa* and *A. rubra*). The clustering of *Betula papyrifera* with the subgenus Alnobetula confirms the closeness of *Betula* to the genus Alnus.

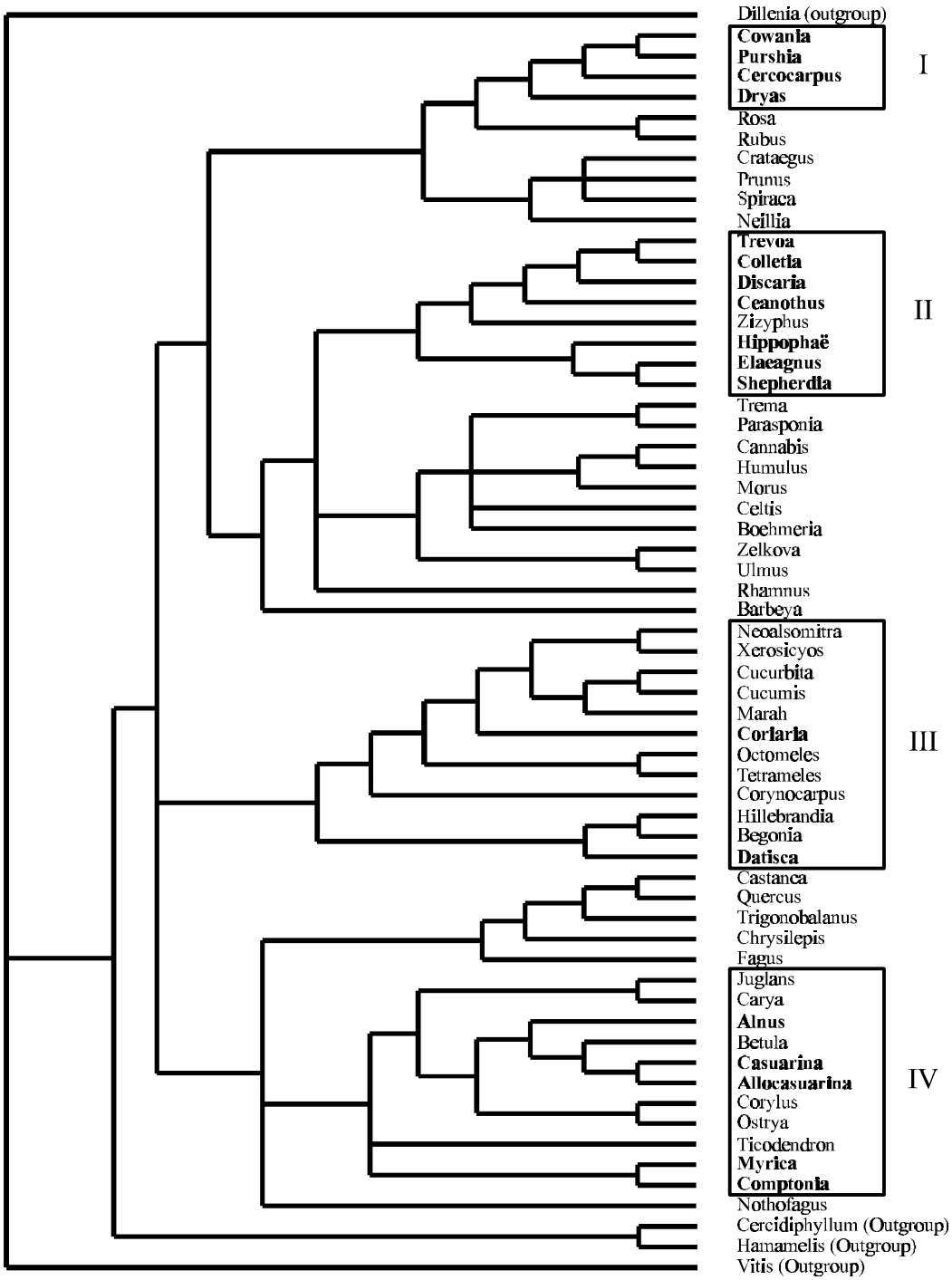


Figure 1 Phylogenetic tree, showing the major clades encountered in an analysis of the *rbc*L gene sequence from actinorhizal plants (in bold) and non-actinorhizal plants (modified after Swensen and Mullin 1997; Used with permission of the publisher). Clades, containing actinorhizal plant species are indicated by boxes, numbered I to IV.

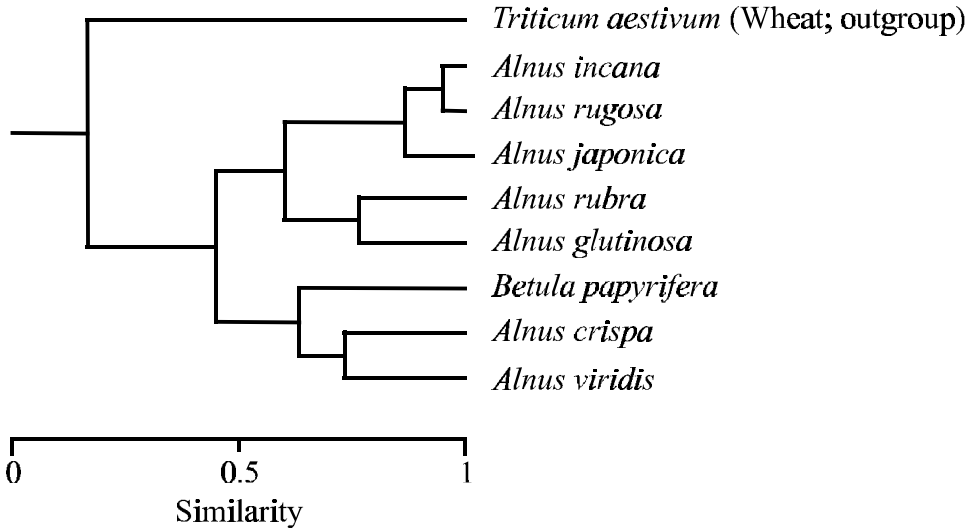


Figure 2 UPGMA dendrogram of eight Betulaceae based on RFLP of the ribosomal DNA region (after Bousquet *et al.* 1989; Used with permission of the publisher).

USE OF NITROGEN-FIXING SYMBIOSES

The positive effect of symbiotic N_2-fixing plants on the growth of nearby farming crops has been recognised separately by various agricultural civilisations. In Europe, this growth-enhancing effect was found when cultivating crops on fields where earlier beans had been grown. Even now, planting beans for soil fertilisation is common practice in various forms of sustainable agriculture. A similar growth-enhancer is the alder, of which an old English rhyme says "*The alder, whose fat shadow nourisheth, Each plant set neere him long flourisheth*". In other parts of the world native people, like the South-American Indians and the Japanese, had recognised similar beneficial effects with certain indigenous trees and shrubs, and these plants were used extensively as windbreaks, growth-enhancers, soil stabiliser and source of lumber (Dawson 1986, Míguel *et al.* 1978, Paschke 1997, Uemura 1971). Nowadays, nitrogen-fixing plants are still useful, although in many cases they have been replaced by industrially produced nitrogen fertilisers. Leguminous plants obviously remain an important source of

food for both man and cattle. Even though a plant like *Datisca cannabina* is no longer interesting for the production of natural dyes, it is still a useful candidate for stabilisation of steep hills (Hafeez *et al.* 1984b). *Casuarina* species are widely used as a source of timber and firewood, and actinorhizal plants may be employed in forestry as nurse crops (Binkley *et al.* 1984, Bollen *et al.* 1967, Carpenter and Hensley 1979, Dawson 1986, Paschke *et al.* 1989), although the costs of maintaining actinorhizal nurse plants are often overlooked (Akkermans and Hahn 1990).

As the symbiotic systems of plants with *Rhizobium* and *Frankia* are important with respect to nitrogen fixation (Thomas and Berry 1989, Torrey 1978), these interactions have been the focus of extensive studies. The objective of this chapter is to provide a comprehensive introduction to the symbiosis of *Frankia* with higher plants, and specifically with *Alnus glutinosa* (L.) Gaertn., the Black Alder.

FRANKIA ASSOCIATED WITH PLANTS

Symbiotic *Frankia* are located in specialised structures, or nodules, along the root system of the host plants. The occurrence of *Frankia* inside symbiotic root nodules was first reported in 1866 by Woronin, who described hyphae and vesicles and stated that these belonged to a parasitic fungus. Later, these hyphae were correctly identified as belonging to an actinomycete (Becking *et al.* 1964, Lechevalier and Lechevalier 1979), and currently, *Frankia* is considered to belong to the order *Actinomycetales*, family Frankiaceae, genus *Frankia* (Becking 1970), together with the related genus *Geodermatophilus* (Hahn *et al.* 1989b). The root nodules are analogous to those induced by *Rhizobium* in legumes, and they provide an environment where *Frankia* can grow and prosper, while providing the host-plant with fixed nitrogen. Through microscopic studies (Berry 1984), and subsequent molecular biological studies (Guan 1997, Pawlowski *et al.* 1996, Ribeiro 1996, Van Ghelue 1994) the infection process, nodule formation and colonisation by *Frankia* has become better understood.

Infection of the host

The actual process of root infection by symbiotic bacteria starts with the chemotaxis, and subsequent attachment of the bacterial cells to the surface of the plant. *Rhizobium* is thought to bind to its host through a specific interaction between plant-secreted lectins and specific carbohydrates on the bacterial cell surface (Bohlool and Schmidt 1974). In the case of *Frankia*, the exact mechanisms of recognition and binding are still unclear, but there is a possibility that here too, lectins are involved in the process (Chaboud and Lalonde 1983).

Like *Rhizobium*, in some host species *Frankia* infects the root system by penetrating root hairs (Berry *et al.* 1986, Berry and Torrey 1983). Prior to infection host root hairs grow deformed, getting twisted and branched and often form bulging tips. After this, *Frankia* hyphae may or may not penetrate the root hair cell wall. It has been demonstrated that initiation of root hair deformation by *Frankia* is triggered by plant exudates (Van Ghelue 1994, Van Ghelue *et al.* 1997). Here again, a similar mechanism was described for *Rhizobium*, which only expresses its nodulation (*nod*) genes after induction by specific flavonoids produced by the host plant (Peters *et al.* 1986, Relic *et al.* 1994). The activation of these *nod* genes results in the excretion of Nod factors (lipo-oligosaccharides) by the bacterium (Fisher and Long 1992). The Nod factors subsequently initiate root hair deformation and cortical cell divisions (Djordjevic *et al.* 1987). While no *Frankia nod* genes have yet been found, for actinorhizal symbioses a similar signal exchange sequence is supposed. It is certain that flavonoid compounds, like with *Rhizobium*, can have a significant impact, both positive and negative, on the nodulation of alder by *Frankia* (Benoit and Berry 1997).

The fact that root hair curling is an important step in nodule development was confirmed by Callaham *et al.* (1979), who reported that nodule formation was positively correlated with the extent of root hair deformation on the host plant. In principle, only one root hair infection is needed, but in practice several root hair infections can be involved in the induction of one nodule (Diem *et al.* 1983).

Before *Frankia* actually enters the plant root, a pre-nodule is formed, which can be recognised as a slight local swelling of the root (Angulo Carmona 1974). Pre-nodule formation is induced by stimulation of cortex cell division near the infection site, possibly

through excretion of plant hormones by *Frankia* (Berry *et al.* 1989, Miguel *et al.* 1978, Wheeler *et al.* 1984).

A second mode of infection, also employed by *Rhizobium*, does not involve the plant root hairs, but proceeds by direct intercellular penetration of the root epidermis, and colonisation of the intercellular spaces of the root cortex. This method of infection is primarily found in symbiotic relationships between frankiae and members of the Elaeagnaceae (Miller and Baker 1985), but is also known to occur in other actinorhizal plant families, with the exception of the Betulaceae, Casuarinaceae and Myricaceae. Which type of infection is used seems to be largely determined by the host plant, as is indicated by the observation of a particular *Frankia* using both modes of infection, depending on the host species (Miller and Baker 1986). Bond (1959) and co-workers (i.e. MacConnel and Bond 1957) reported that nodulation of several actinorhizal plant species by *Frankia* is inhibited by a high nitrate concentration. Kohls and Baker (1989) found evidence that induction of root hair formation is also suppressed. This might explain why nodulation of plants, infected via epidermal cells, such as *Elaeagnus* species, was unaffected by the substrate nitrate level (Kohls and Baker 1989). Unlike the root hair infection mode, the epidermal root infection does not initiate pre-nodule formation before the actual nodule primordium is formed.

After initial extra-cellular growth (in the case of epidermal root infection), *Frankia* eventually penetrates the host cells. When entering a plant cell (immediately, in the case of root hair infection), *Frankia* is embedded into a layer of cell wall-like material (Berry and Torrey 1983), surrounded by a pectinaceous matrix (Lalonde and Knowles 1975) This creates a tubular encapsulation, functionally similar to the infection thread described for the *Rhizobium*-legume symbiosis (Berry and Sunell 1990). It is suggested that this matrix material can also be used as a substrate by the colonising frankiae, because hyphae imbedded in the matrix are surrounded by a clear dissolution zone. *Frankia* is known to possess pectinolytic enzymes (Séguin and Lalonde 1989, Simonet *et al.* 1989) which may be used to facilitate the infection process.

Nodule formation

The actual nodule is not initiated in the pre-nodule, but in the root pericycle. The *Frankia* hyphae invade the root pericycle from the pre-nodule cells, penetrating cortical cell walls in the process. In the infected region lateral root initiation is stimulated by the endosymbiont (Angulo *et al.* 1975). This new root primordium is the basis of the new root nodule, and possesses a central vascular cylinder, just like a normal lateral root. Around this vascular tissue, cortical cells infected with *Frankia* are situated. In contrast, in legumes the infected nodule cells are situated in the centre of the root nodules, surrounded by cortex cells and vascular tissue. So, instead of the lateral root morphology of actinorhizal nodules, legume nodules have a stem-like appearance. An exception forms the *Rhizobium-Parasponia* symbiosis, whose nodules closely resemble actinorhizal nodules in structure and in development (Trinick 1979). The infection of an actinorhizal nodule seems to be co-ordinated with considerable expansion of the infected host cells which gives rise to the swelling of the root nodule. Within these nodule cortical cells, *Frankia* hyphae branch and multiply, while the host cells surround them with a plasma membrane and cell-wall material, like in the root hair cells. Again, this cell wall material might be utilised by certain *Frankia* as a substrate, since some strains possess pectinase (Simonet *et al.* 1989, Séguin and Lalonde 1989) and cellulase (Safo-Sampah and Torrey 1988). The presence of pectinase (*pel*) genes is not uncommon in other soil microorganisms, and also *Rhizobium* has the ability to degrade pectins (Angle 1986).

The apical meristeme is not infected, and only newly formed cortical cells are invaded by the endosymbiont. In general, the nodule displays extensive dichotomous branching, producing the characteristic multi-lobed appearance of actinorhizal nodules. In some host genera, non-infected roots are formed at the tips of the nodule lobes, and with their negatively geotropic growth seem to facilitate the transport of additional oxygen into the root nodules (Tjepkema 1977, Torrey and Callaham 1977). Although the nitrogen-fixation process itself is inhibited by high oxygen levels, the vegetative hyphae and the host cells still require oxygen for proper growth. It has been shown that lack of oxygen reduces nodule development in the root system of alder plants (Kurdali and Domenach 1991, MacConnell

1959). This is also illustrated by the complete absence of nodulation of *Alnus glutinosa* in swamps at soil depths devoid of oxygen (Akkermans 1971, Akkermans and Van Dijk 1976).

While free nitrate levels can affect nodule formation through root hair suppression, stored internal nitrogen levels of the host plant can also regulate nodulation (Thomas and Berry 1989). Furthermore, an active, systemic mechanism for feedback control of nodulation prevents excessive nodulation of the root system of some actinorhizal host plant species (Dobritsa and Novik 1992, Wall and Huss-Danell 1997). While in these host plants nodulation proceeds normally, a second incubation with *Frankia* showed a markedly retarded nodulation response, which extended to parts of the root system that were not nodulated previously. Such a feedback control system for nodulation is also known to exist in *Rhizobium*/legume interactions (Caetano-Anollés and Gresshoff (1991). In this way the host plant may create a balance between costs and benefits of the symbiosis, its nitrogen requirements and the nitrogen-fixation of the nodules (Dobritsa and Novik 1992). In a way, this feedback mechanism has much in common with the phenomenon of induced resistance of plants (Gatz 1997, Karban *et al.* 1997) and is mirrored by the balance between resistance and tolerance of a plant like *Arabidopsis thaliana* against plant herbivory (Mauricio *et al.* 1997).

Differentiation of *Frankia*: Vesicles

Intracellular hyphae undergo extensive branching, and eventually vesicles — terminal swellings from short lateral hyphae — are formed. These vesicles seem to be closely associated with nitrogen fixation, as their absence also marks the absence of nitrogen fixation, both in culture (Tjepkema *et al.* 1980) and in nodules (Mian and Bond 1978). By using immuno-gold labelling techniques on ultra-frozen microtome slices of cell pellets, it was shown that, indeed, nitrogenase activity is solely restricted to the vesicles (Meesters *et al.* 1987). Earlier results already indicated that in the vesicles the redox potential is sufficiently low for nitrogen fixation to occur (Akkermans 1971). As with the exact means of *Frankia* infection (i.e. root hair infection or root epidermis penetration), the shape and arrangement of the vesicles inside infected cells are controlled by the host plant species (Lalonde and Calvert 1979). *Alnus* and *Elaeagnus*-type nodules contain large, spherical, and septate vesicles, *Dryas* species contain elliptical, non-septate vesicles, *Coriaria* (Allen *et al.* 1966), *Myrica*

and *Comptonia* nodules have club-shaped vesicles, whereas in members of the genus *Casuarina* vesicles are absent, and symbiotic nitrogen fixation takes place in the hyphae (Berg and McDowell 1987, Sellstedt and Mattson 1994).

Microscopic studies show that the multi-laminate cell envelope surrounding the vesicles of *Alnus*-nodulating *Frankia* strains resembles the glycolipid membranes found associated with heterocysts of cyanobacteria (Torrey and Callaham 1982). With these *Frankia* strains, this cell-envelope forms a diffusion barrier to oxygen, thus protecting the nitrogenase located inside the vesicles (Murry *et al.* 1984, 1985). Parsons *et al.* (1987) found that the thickness of the envelope can be adapted to function as an oxygen barrier under a wide range of pO_2 levels, to a maximum of up to 3 times the atmospheric concentration. Abeysekera *et al.* (1989) suggested, however, that this oxygen diffusion barrier might not be the only O_2 protection mechanism in *Alnus* nodules (nodule O_2-respiration; Huss-Danell *et al.* 1987, haemoglobin; Suharjo and Tjepkema 1995). In some other actinorhizal genera, it is primarily the host plant that provides the necessary oxygen diffusion barrier (plant cell wall in *Myrica*: Zeng and Tjepkema 1994; *Casuarina*: haemoglobin; Gherbi *et al.* 1997, discontinuity of intercellular air spaces; Tjepkema and Murry 1989, Zeng *et al*. 1989; nodule periderm in *Coriaria*: Silvester and Harris 1989). Even so, the nitrogenase activity in many actinorhizal plant species is rather dependent on the ambient oxygen concentration (Tjepkema 1979).

Differentiation of *Frankia*: Sporulation

Along with the vesicles, in some actinorhizal plant species (Torrey 1987), ageing hyphae may form structures, functionally analogous to sporangia. These structures, spindle-shaped in pure cultures and in nodules from plants in hydrocultures, usually emerge when formation of vesicles is initiated.

Mature sporangia are especially abundant in ageing, or dead host cells in which the endosymbiontic vesicle stadium is already degenerating (Becking *et al.* 1964, VandenBosch and Torrey 1985). Sporangia are formed by specific cell-divisions, preferably in thick hyphae, accompanied by septation perpendicular to, and later also parallel to the longitudinal axis (Van Dijk and Merkus 1976).

With respect to spore-formation, within *Frankia* two groups are recognised (Van Dijk 1979). One type, named sp(-), forms spores in pure cultures, but fails to do so inside root nodules. The other strain type, sp(+), is comprised of organisms which form spores within the root nodule symbiosis, but which have until now eluded every attempt to isolate and maintain them in pure culture. This division of *Frankia* in sp(+) and sp(-) strains may not be absolute, as strains possessing intermediate nodular spore-formation have been reported (Van Dijk *et al.* 1988). Nonetheless, for many actinorhizal symbioses the distinction between sp(+) and sp(-) seems to be valid (Torrey 1987). Finally, Simonet *et al.* (1994) demonstrated that sp(+) and sp(-) *Alnus*-infective nodules can be separated on the basis of their respective bacterial 16S rDNA sequences, which suggests that these two types of endophytes are genetically different.

Nodulation experiments have shown that whereas the sp(+) strains in general have a much higher infection potential than the sp(-) strains (Akkermans and Van Dijk 1976, Houwers and Akkermans 1981, Van Dijk 1984), this improved infectivity may also have a price. Several authors reported reduced acetylene reduction activity by nitrogenase in sp(+) nodules compared to sp(-) (Monz and Schwinzer 1989, Normand and Lalonde 1982, VandenBosch and Torrey 1984, Weber 1990), a higher energy requirement of the nitrogenase (Monz and Schwintzer 1989) and reduced vesicle longevity (VandenBosch and Torrey 1985). On the other hand, MacKay *et al*. (1987) and Van Dijk (1978) found no differences in nitrogen fixation capacity between sp(+) and sp(-) nodules, and Kurdali *et al.* (1989) reported a strong influence of soil type on the effectivity of both sp(+) and sp(-) *Frankia* strains. While the discrepancies between the results of these experiments may seem strange, it is important to realise, that the genetic make-up of the host plants, and indeed of the particular *Frankia* strains that were used, may have considerably influenced the outcome of the nitrogen fixation measurements (Kurdali *et al.* 1990).

In Finland, sp(+) nodules were mainly found with *Alnus incana*, and sp(-) nodules were predominantly found on *Alnus glutinosa* (Weber 1986). In other ecosystems studied, mixed sites with both spore types are often encountered (Kashanski and Schwintzer 1987, Van Dijk 1978, 1984), as are sites having only sp(-) nodules (Kashanski and Schwintzer 1987). Generally, sp(+) nodules are found under established actinorhizal plant vegetations while sp(-

) *Frankia* can be encountered in pioneer ecosystems (Holman and Schwintzer 1987, Van Dijk *et al.* 1988), or even in soils without a host plant vegetation (Paschke and Dawson 1992a). This has fuelled the belief that sp(+) *Frankia* form a group of obligate symbionts, while sp(-) strains can also grow as saprophytes. Also, sp(+) type *Frankia* have been much more resistant to isolation than sp(-) strains (Quispel and Tak 1978) and even now, no proven sp(+) *Frankia* isolates are successfully maintained in pure culture.

Spores do not seem to be more resistant to extreme soil conditions (i.e. high temperature) than the vegetative hyphae, so probably their only function is to enhance the dispersal of *Frankia* in the soil (Van Dijk 1984).

Nodule physiology

The symbiotic relationship between *Frankia* and its host is an intricate system, in which host cell functions are tightly interwoven with the endosymbiotic cellular activities. Recently, molecular evidence for this has been uncovered in the form of plant-derived nodule-specific genes in *Alnus glutinosa* (Guan *et al.* 1997, Pawlowski 1997, Ribeiro *et al.* 1995, Ribeiro 1996). Cortical cells are newly formed in the apical nodule meristem, and are subsequently infected by *Frankia* hyphae. Towards the base of the nodule, the infected cells become progressively older, and the endosymbiont shows signs of senescence (degeneration of hyphae and vesicles; in sp(+) nodules increase in sporangium formation and size). This development from pre-infection cortical cell, infected cell, to senescent cortical cell is paralleled by expression patterns of these nodule-specific genes (Ribeiro 1996), and of *nif* gene expression (Pawlowski *et al.* 1995).

The commitment of the endosymbiont to symbiotic nitrogen fixation is shown by research on the carbon metabolism of *Frankia* inside the root nodules of *A. glutinosa* and *H. rhamnoides* (Huss-Danell *et al*. 1982a). Enzymes of the glyoxylate cycle were not detected in vesicles of the *Frankia* strain that was tested. In contrast, their occurrence was demonstrated in *Frankia* grown in liquid culture media.

The host controls the assimilation of the fixed nitrogen. Research on *Alnus* has shown that both glutamine synthetase and glutamate dehydrogenase, and the enzyme ornithine carbamoyl transferase are abundant only in nodule host tissue (Blom 1982, Blom *et al.* 1981, Gardner and Scott 1982). These enzymes are needed in *Alnus* for the assimilation of fixed nitrogen (ammonia), by forming glutamine and citrulline, respectively. Likewise, glutamine synthetase activity was only found in vegetative hyphae of pure cultures of *Frankia* strain CpI1 (Noridge and Benson 1986), and not in the vesicles. Thus, most of the ammonia formed by the bacteria inside the root nodules would be available to the plant, keeping the endosymbiont starved for nitrogen. Results obtained by Akkermans (1971) already pointed in this direction: $^{15}N_2$, offered to a *Frankia*-host symbiosis was largely incorporated in reduced N outside the vesicle fractions after a short incubation time.

In temperate zones, nitrogen fixation activity by *Alnus glutinosa* nodules ceases in winter (Akkermans 1971). Nodule dormancy seems to be associated with the levels of abscisic acid (Watts *et al.* 1983), which may act by repressing nodule auxin activity. In spring, nitrogenase activity is slowly reinitiated (probably from newly formed vesicles) when the plant starts budding, until increased availability of photosynthates stimulates higher rates of nitrogen fixation (Akkermans 1971, Wheeler *et al.* 1983). Fluctuations in nitrogenase activity are the result of variations in photosynthetic activity of the plant (Huss-Danell *et al.* 1989) and of soil water-table fluctuations. Changes in combined nitrogen content of the soil (Granhall *et al.* 1983, Huss-Danell *et al.* 1982b) may also inhibit nitrogen fixation, but in some cases additional nitrogen fertilisation is beneficial to the symbiosis (Steele *et al.* 1989).

In general, nitrogen fixation and *Frankia* survival *in planta* are less affected by adverse environmental conditions than nitrogen fixing and survival of the free-living endophyte are (Hennessey *et al.* 1989), attesting to the protective buffering capacity of the plant tissues enveloping the endosymbiont.

FRANKIA OUTSIDE NODULES

Isolation and *in vitro* growth requirements

Initially, *Frankia* were believed to be obligate symbionts, due to the consistent failure of all isolations. Only in 1978 the first *Frankia* isolate became available (Callaham *et al.* 1978), and even today *Frankia* strains from some actinorhizal genera still await isolation. Isolation techniques (see Stowers 1987 for a review) that have been used to successfully isolate *Frankia* from nodules are serial dilution (Diem *et al.* 1982), micro-dissection (Berry and Torrey 1979), OsO_4 surface sterilisation (Normand and Lalonde 1982), filter exclusion (Benson 1982, Weber *et al.* 1988), and sucrose density fractionation (Baker and O'Keefe 1984, Baker and Torrey 1979, Burggraaf *et al.* 1981). All isolated strains are slow-growing, aerobic heterotrophs, with doubling times ranging from about 15 to 48 h (Benson and Silvester 1993, Savouré and Lim 1991, Schwencke 1994).

In vitro carbon requirements

Although some *Frankia* strains can utilise glucose as a carbon-source (Carú 1993), many of the strains that have been isolated grow primarily on acetate, pyruvate or propionate (Akkermans *et al.* 1983). Growth on low molecular-weight fatty acids seems to be a universal trait of *Frankia* (Akkermans *et al.* 1981, Shipton and Burggraaf 1982), whereas some *Frankia* strains are also able to use other carbohydrates (Lechevalier *et al.* 1983), and various Tweens (Blom 1981) as a carbon and energy source. Furthermore, several lipid supplements have been shown to positively affect *Frankia* growth rate *in vitro* (Lalonde and Calvert 1979, Schwencke 1991), although much of the growth stimulation may be due to a beneficial effect on the bacterial plasma membrane, rather than on the cell's carbon metabolism (Selim *et al.* 1996, Selim and Schwencke 1994). Certain plant-extracts facilitated *Frankia* isolation (Quispel and Tak 1978, Quispel *et al.* 1983) or growth (Ringø *et al.* 1995, Rogers and Wollum 1974), and the active component in lipid petrol extracts from *Alnus*-roots was characterised as dipterocarpol (Quispel *et al.* 1989). The inducing effect on outgrowth of *Frankia* from nodules by these lipid components may simply reflect the transition of the endophyte physiology from a symbiotic to a free-living mode, whereas the

growth enhancement by phenolic compounds could indicate that such compounds function as chemical mediators between the host cell and the symbiotic *Frankia* (Perradin *et al.* 1983). Several strains produce extracellular protease and amylase which may play a role in the infection process (Lechevalier *et al.* 1983).
Possible storage compounds are glycogen and trehalose, of which the latter one may also have a function of tolerance against desiccation in *Frankia* (Burleigh and Dawson 1994).

In vitro nitrogen requirements and N_2 fixation

In absence of nitrogen fixation, *Frankia* isolates can utilise a wide variety of nitrogen sources, including NH_4^+, NO_3^- and a number of amino acids (Akkermans *et al.* 1983, Blom *et al.* 1981, Shipton and Burggraaf 1982). In many strains, vesicles are formed in culture media without combined nitrogen (Tjepkema *et al.* 1981). Exceptions to this are strains such as Cc1.17 (Meesters *et al.* 1985). Nitrogen fixation activity rapidly declines in batch cultures, which may be partly caused by accumulation of inhibitory waste products. In line with this, continuous-culture grown *Frankia* show a stable nitrogenase activity (Harris and Silvester 1992).

In vitro abiotic growth parameters

Although some *Frankia* are considered to be strictly microaerophilic (Lechevalier *et al.* 1983), this may not hold true for all strains, as *in vitro* growth can be significantly increased through stirring (Schwencke 1991). In static culture the protein content of *Frankia* strain ArI3 doubled in about 30 days, whereas in a stirred fermentor the doubling time was about 2.5 days (Ringø *et al.* 1995). Furthermore, *Frankia* does not grow under totally anoxic conditions (Benson *et al.* 1985, Callaham *et al.* 1978).
Although a salt-tolerant strain has been found (Girgis *et al.* 1992), most *Frankia* strains cannot grow under extreme high osmotic conditions such as some other actinomycetes (Shipton and Burggraaf 1982). The ranges of temperatures (10-40°C) and pH (5.5-8.0) (Burggraaf and Shipton 1982) over which growth takes place are rather unexceptional for a soil organism.

TAXONOMY

Host specificity groups

Originally, species-name assignment was based on the host plant of the *Frankia* in question (Becking 1970), but this was shown by Baker (1987) to be an oversimplification. Becking (1968) introduced the concept of cross-inoculation, or host-specificity groups, which was extended by Baker (1987). In nodulation experiments, *Frankia* strains were shown to be able to form effective symbioses with all host plants within such a host-specificity group, but were unable to do so with host plant species associated with the other specificity groups (Bermúdez de Castro *et al.* 1976). Differences in the composition of the cell-wall surfaces of *Frankia* strains belonging to the *Alnus* and *Hippophaë* specificity groups confirmed the validity of this subdivision (Baker *et al.* 1981, Chaboud and Lalonde 1983, Gardes and Lalonde 1987). Host specificity group 1 includes *Frankia* strains capable of forming nodules with *Alnus* (Baker and Torrey 1980, Horrière 1984), *Comptonia*, and *Myrica*. Host specificity group 2 contains strains, nodulating *Casuarina*, some *Allocasuarina*, and *Gymnostoma*. Host specificity group 3 *Frankia* strains nodulate *Elaeagnus*, *Hippophaë*, and *Shepherdia*. Some plant species, belonging to the genera of *Myrica* and *Gymnostoma*, can be nodulated by a wide variety of *Frankia* strains, and are called promiscuous hosts (Baker 1987, Racette and Torrey 1989). Others, like *Allocasuarina lehmanniana*, seem very selective in their acceptance of infective *Frankia* strains (Torrey and Racette 1989). Host specificity group 4 consists of strains, able to form symbiotic interactions with host species from the plant genera of group 3, but unable to nodulate the promiscuous hosts. Also from the point of view of the *Frankia* strains, the boundaries between the specificity groups are not absolute (Bosco *et al.* 1992). It must be stated that this picture can change in the future, and more host specificity groups will be added, as several actinorhizal plant genera, (Rhamnaceae: Gauthier *et al.* 1984) have not been (fully) tested or have yet to yield infective *Frankia* isolates.

Although the host-specificity groups form a useful tool for classification of the various *Frankia* strains, they do not provide enough detail to come to a successful species definition.

Molecular characterisation of *Frankia*

Molecular biological techniques have been a useful and potent addition to the traditional physiological tests (Horrière 1984, Lechevalier and Ruan 1984), for the detection of and distinction between *Frankia* strains, whether *in vitro* or *in planta*. These techniques include fatty acid analysis (Mirza *et al.* 1991), SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) protein pattern analysis (Benson and Hanna 1983, Gardes and Lalonde 1987), isozyme variations (Gardes *et al.* 1987), RFLP (Restriction Fragment Length Polymorphism) analysis (Dobritsa 1985, Maggia *et al.* 1992, Nittayajarn *et al.* 1990, Simonet *et al.* 1989), DNA- hybridisation (Simonet *et al.* 1988), PCR amplification (Hahn *et al.* 1989a, Simonet *et al.* 1991), and RNA and DNA sequence analysis (Harry *et al.* 1991, Nazaret *et al.* 1991). Although these techniques allow for the identification of separate groups within *Frankia*, even within host-specificity groups (Benson *et al.* 1984), there is still much discussion about the attributes, which have to be used for *Frankia* species definition (Lalonde *et al.* 1988). An additional complication in taxonomic subdivision is the large strain diversity which has been shown to be present when using the techniques mentioned above (Faure-Raynaud *et al.* 1991, Gardes *et al.* 1987). Presently, isolates of *Frankia* are only characterised by international strain codes, which specify the culture collection in which the strain is kept, and the genus and species of host plant it was isolated from (Lechevalier 1983).

Although no species definition has been yet agreed upon, the phylogeny of *Frankia* is being studied. The combination of PCR amplification and sequence analysis makes it possibly to analyse *Frankia* strains without prior isolation. Using 16S rRNA and 23S rRNA gene sequences, phylogenetic trees have been constructed (Hahn *et al.* 1989b, Hönerlage *et al.* 1994, Normand *et al.* 1996). While these trees are comprised of a limited number of *Frankia* strains, and do not form a complete consensus, phylogenetic techniques show a great potential for the future of *Frankia* taxonomy.

ECOLOGY

Dispersal of *Frankia*

As nodules are part of the plant root system, they have the same life span as the individual roots. Under field conditions root nodules in the alder rarely exist more than ten years. New roots and nodules are formed almost continuously during the life of an actinorhizal plant, and the average turnover frequency of root nodules of a tree species such as *Alnus glutinosa* is about 3 years (Akkermans 1971, Akkermans and Van Dijk 1976, Van Dijk 1984). When root nodules die off, *Frankia* particles are released into the soil. Thus, the senescence and degeneration of root nodules can be an important factor (especially for sp(+) strains) in the release, and maintenance of a large *Frankia* population in the soil of ecosystems containing host-plants (Van Dijk 1984).

There may be mechanisms, other than the degeneration of root nodules, which can lead to the dispersal of *Frankia*. While wind-borne dispersal is unlikely (Arveby and Huss-Danell 1988), this cannot be said from water movement in wet sediments, or from rainwater runoff down terrain elevations. Especially spores could, due to their small size and large numbers, contribute to the spreading of *Frankia* in an ecosystem. There also is some evidence for the role of soil fauna (e.g. earthworms) in the dispersal of *Frankia* (Reddell and Spain 1991).

Growth and persistence in soil

The possibility of (extranodular) saprophytic growth of *Frankia* in the root systems of actinorhizal and non-host plants has been mentioned previously. Considerable numbers of *Alnus*-infective *Frankia* strains of the sp(-) strain type, have been detected in the rhizosphere of *Betula* spp. (Paschke and Dawson 1990, Smolander and Sundman 1987, Smolander 1990a, 1990b, Smolander and Sarsa 1990, Van Dijk 1984), *Populus* sp. and grasses (Houwers and Akkermans 1981). *Alnus* and *Betula* are taxonomically closely related genera in the Betulaceae, and it has been suggested (Paschke and Dawson 1992b, Zitzer and Dawson 1992) that because of this, *Frankia* can also grow as a saprophyte in the rhizosphere of *Betula* spp. Rönkkö *et al.* (1993) have confirmed that *Frankia* can colonise root systems

of grasses (*Poa pratensis*, *Festuca rubra*). Likewise, Zimpfer *et al.* (1997) also reported the presence of *Frankia* in soils devoid of host plants.
When growing saprophytically, *Frankia* may fulfil the role of a root-associated nitrogen fixer, and its positive effect on plant growth might be augmented by its ability to produce auxins (Wheeler *et al.* 1984).

Detection of *Frankia* populations in soil can be achieved through biotests, i.e. using host plant nodulation to enumerate the number of nodulating units (NU) (or nodule forming capacity, NC) per volume of soil (Huss-Danell and Myrold 1994, Van Dijk 1978). PCR amplification of specific *Frankia* sequences from soil-extracted DNA can give the number of *Frankia* genomic units (GU) per volume of soil (Picard *et al.* 1992). Essentially, the NU or NC represents the number of *Frankia* particles, able to form an active symbiosis, while GU represents the total number of *Frankia* particles. Myrold and Huss-Danell (1994) showed that the GU for a particular location was constant, while the NU changed seasonally. The NU per volume of soil can be determined through inoculation of plants with dilution series of soil solutions (Van Dijk 1978), or through inoculations using the MPN principle. Both methods have been compared by Myrold and Huss-Danell (1994) and were found to give similar results. One has to be careful when comparing the PCR-based *Frankia* detection with the biotest assay in terms of yield or sensitivity because these two methods give essentially different information. The PCR-based method quantifies all *Frankia* DNA present, including dormant and dead cells and possibly fossil DNA, while only active, compatible, and nodulating *Frankia* cells will emerge from the bioassays. The soils that were examined by Myrold and Huss-Danell (1994) gave on average 100 times higher GU values than nodulating units.

The number of nodulating units of a soil is strongly correlated with the soil pH (Smolander and Sundman 1987, Smolander *et al.* 1988), and high nodulation capacities were found at soil depths, where moist, but well-aerated conditions prevailed (Dawson *et al.* 1989). This confirmed the findings that *Frankia* is rather sensitive to water-stress (Shipton and Burggraaf 1982), which has also been found for the nodulation process itself (Pratt *et al.* 1997). Nalin *et al.* (1997) demonstrated that the *Frankia* population composition could change markedly

with soil depth and with a decrease in the soil organic matter content. Consistent with this, a decrease of *Frankia* nodulation was found with increasing soil depth (Zitzer *et al.* 1996).

Diversity in soils and nodules

There exists a large amount of indirect evidence for the occurrence of co-infections of two or more *Frankia* strains in one nodule (Benson and Hanna 1983, Dobritsa and Stupar 1989, Hafeez *et al.* 1984a, Murry *et al.* 1997, Reddell and Bowen 1985, Simonet *et al.* 1994). This phenomenon might very well be dependent on the density of *Frankia* around the infected host plant, instead of being a deliberate infection strategy. Local strain diversity can be significant (Chaudhary and Mirza 1987, Nalin *et al.* 1997, Zitzer and Dawson 1992), but may also be small (Maggia *et al.* 1992, Simonet *et al.* 1994). According to Jamann *et al.* (1992) the extent of *Frankia* strain diversity seems to be unaffected by soil- or geographic factors. Nalin *et al.* (1997), however, found that the composition of the *Frankia* soil community changes with soil depth, which correlated with a decrease in the organic matter content of the soil. Thus, it is altogether possible, that the existence of (micro) gradients in the soil promotes diversity of the *Frankia* population. On a macro-scale, an observer would not perceive such gradients, nor would any differences in the composition of the *Frankia* population be detected.

That *Frankia* is not the only microbial inhabitant of root nodules has been shown by several isolation trials with surface-sterilised nodules. These yielded a large variety of microorganisms, primarily actinomycetes, which had to be co-infectants or co-symbionts, as they were unable to nodulate the host plants themselves (Allen *et al.* 1966, Niner *et al.* 1996, Ramírez Saad *et al.* 1998, Uemura 1952).

Interactions with soil organisms

The rhizosphere forms a heterogeneous ecosystem, where a large variety of micro-organisms exist together. It has been shown that many of these microorganisms interact with *Frankia* and/or its host. Some soil organisms, like *Pseudomonas cepacia* (Knowlton *et al.* 1980, Knowlton and Dawson 1982) facilitate the infection by *Frankia* of the host root system, probably through induction of root hair deformation. Rojas *et al.* (1992) describe

how several soil actinomycete isolates had a positive effect on *Frankia* acetylene reduction rates with *Alnus rubra*, while other actinomycete strains had a negative effect on the growth of the host plant. Probanza *et al.* (1996) found positive effects of some *Bacillus* spp. on the growth of alder, whereas some *Pseudomonas* spp. negatively influenced alder development. Endo- (Gardner *et al.* 1984, Russo 1989) and ecto-mycorrhiza (Chatarpaul *et al.* 1989) both have a positive, even synergistic effect on the host / endophyte interaction. Also, there are microorganisms that are able to produce non-actinorhizal "pseudo-nodules" on the roots of alder (Berg *et al.* 1992, Savka *et al.* 1992). Some pseudo-nodules have an appearance quite similar to *Frankia*-induced nodular structures, as is the case with the myco nodules produced by some *Penicillium* species (Van Dijk 1979, Sequerra *et al.* 1995, Valla *et al.* 1989). They can appear in high numbers on the roots of alder seedlings (Van Dijk 1979). Also, the mode of infection is rather similar to the root hair infection utilised by *Frankia*, although infection is limited to the root cortex (Sequerra *et al.* 1994).

Plant pathogenic nematodes may interact with *Frankia* nodulation in soil, due to their detrimental effect on the root system of the host plant (Zoon 1995). With respect to the host plant, *Frankia* strains also interact with each other. Hahn *et al.* (1990b) reported that one (non-nodulating) *Frankia* strain increased nodulation of the host root system by a second *Frankia*. Furthermore, it was reported that mixed *Frankia* inoculates gave better growth of *Alnus* spp. than the best individual *Frankia* strain (Prat 1989).

Variation in *Frankia* host compatibility

The *Frankia*/host plant interaction is the result of a complex inter-species process, and varying degrees of compatibility may exist between the two symbiotic partners. When a *Frankia* strain and an actinorhizal plant are totally compatible, the combination will result in an optimal symbiotic interaction (Hooker and Wheeler 1987). When endophyte and plant are totally incompatible, no nodulation will occur at all (Zhongze *et al.* 1984). This all-or-nothing principle is the basis for the host-specificity groups described above. With some *Frankia*/host combinations, nodules with reduced nitrogen-fixing capabilities (Domenach *et al.* 1989) are formed, while others give rise to totally ineffective (non-nitrogen fixing) nodules (Bosco *et al.* 1992, Nazaret *et al.* 1989). The nodules resulting from this crippled interaction usually

remain small (VandenBosch and Torrey 1983, Mian and Bond 1976), and vesicles are not found, or occur only in very small numbers (VandenBosch and Torrey 1983). This partial incompatibility between host and endophyte has also been found with *Rhizobium* (Shantaram *et al.* 1987, Amarger 1981).

Since either partner can enter into an effective symbiosis with a more compatible host plant or *Frankia* strain, these interactions could be called facultatively ineffective with respect to nitrogen fixation. There are *Frankia* strains, however, which seem to be unable to form effective, nitrogen-fixing nodules with any actinorhizal plant. Previously, such *Frankia* strains were referred to as 'atypical', but this term is rather uninformative. Instead, Akkermans and Hirsch (1997) proposed to use a universal set of codes, based on well-defined phenotypic characteristics, like root hair curling (Hac), nodulation (Nod) and nitrogen fixation (Fix). A similar system of nomenclature was already in use for the *Rhizobium*/legume symbiosis. Using this code system, 'atypical' *Frankia* nodules should be referred to as being (Nod^- Fix^-) or (Nod^+ Fix^-). For ease of use, Fix^- *Frankia* strains will hereafter be referred to as ineffective *Frankia* strains.

INEFFECTIVE (FIX^-) *FRANKIA* STRAINS

Ecology of facultatively ineffective interactions

Although most facultatively ineffective interactions were the result of *in vitro* inoculation of endophytes and actinorhizal plants from different host compatibility groups, there is also an example of naturally occurring, and ecologically functional ineffective interaction (Weber 1986, Weber *et al.* 1987). It was found that the indigenous sp(-) population with its much lower nodulation capacity successfully maintained itself in an ecosystem containing sp(+) frankiae. This was possible because the sp(-) strain was capable of forming an effective symbiosis with both of the local hosts *Alnus incana* and *Alnus glutinosa*, while the sp(+) strain produced ineffective nodules with the black alder trees. Thus, in *Alnus* (as in the Casuarinaceae family), complete intragenus (intrafamily) compatibility does not exist. Apart from this example, it is likely that facultative ineffectivity occurs on a very small scale, due to partial incompatibility between one particular host tree and the local *Frankia* strain. It is

known that within established *Alnus* populations there can be considerable genetic variation (Bousquet *et al.* 1986, Hendrickson *et al.* 1993), and it is not unthinkable that differences in compatibility to certain *Frankia* stains exist within such variable host plant populations.

Non nodulating, non nitrogen-fixing (Nod$^-$, Fix$^-$) *Frankia* strains

Several isolated *Frankia* strains were found to be unable to nodulate the original host plant, but could participate in a functional symbiosis with another actinorhizal plant species (Diem *et al.* 1982, Normand and Lalonde 1986). Other isolated strains, however, were unable to induce nitrogen-fixing nodules on any host plant. These ineffective *Frankia* strains were found to be associated with effective nodules from a variety of host plant species, including *Alnus glutinosa* (Hahn *et al.* 1988), *Elaeagnus umbellata* (Baker *et al.* 1979), *Datisca cannabina*, and *Coriaria nepalensis* (Chaudhary and Mirza 1987, Mirza *et al.* 1992).

Ecology of Fix$^-$ *Frankia*/host plant interactions

While all of these *Frankia* strains were isolated from effective root nodules, Van Dijk and Sluimer-Stolk (1990) reported that soil under an *A. glutinosa* vegetation had a high nodulation capacity for ineffective *Frankia*, although no ineffective nodules could be detected on the alder root systems *in situ*. Wheeler *et al.* (1981) do mention the occurrence of small nodules on alder plants in field-situations, but this was not further investigated. Apart from this incidental observation, there is no direct evidence that ineffective *Frankia* strains will infect *A. glutinosa* root systems in the field. Still, the nodule formation induced in greenhouse experiments with hydrocultures and soil planted alder seedlings strongly suggests their natural occurrence.

With the alder-derived ineffective strains, it was found that another type of partial incompatibility with the host plant existed. Some of the *A. glutinosa* plants that were used for inoculation experiments completely failed to generate ineffective nodules, while others showed profuse nodulation (Hahn *et al.* 1988). Van Dijk and Sluimer (1994) showed that this resistance of *A. glutinosa* to nodulation by obligatory ineffective *Frankia* is determined genetically, rather than physiologically. Variable resistance was even encountered within half-sib alder seed-lots. Nodulation experiments with an ineffective *Frankia* strain showed a non-

Poisson distribution, with a high percentage of non-nodulated test plants. Between seed batches, substantial and consistent differences in resistance were found. In contrast, a normal Poisson distribution was found using a local effective *Frankia* strain. While seedling resistance was found with *A. glutinosa*, all *Alnus nitida* plants were (ineffectively) nodulated by the ineffective *Frankia* strain. The speed with which the nodules occurred on the alder seedlings also varied considerably. It was found that this is also host-determined, and that plants can be selected for fast nodulation.

While it has not been reported as such, this phenomenon of variable resistance to nodulation by ineffective *Frankia* strains may also occur with other host plant species (Baker *et al.* 1979) and with facultatively ineffective interactions (Torrey and Racette 1989).

As the report by Van Dijk and Sluimer-Stolk (1990) is the only one mentioning the occurrence of ineffective *Frankia* in the soil, it is difficult to assess what is the ecological niche of these organisms in the soil. The ineffective *Frankia* strain type was present in much larger quantities than the local effective strain, as was shown by the nodulation potentials of both strains. Furthermore, the ineffective strain seemed to be present most frequently in the wettest parts of their sampling site, and its nodulation capacity was negatively correlated with the elevation of the soil surface. It is unknown whether this was due to the soil humidity itself, or to related soil parameters.

OUTLINE OF THE THESIS

In conclusion, our knowledge about the ecology of the ineffective *Frankia* strain type, associated with *A. glutinosa* is far from complete. From an ecological point of view a number of questions can be raised:

1) Do ineffective *Frankia* form a common part of the *Frankia* soil population at wet alder sites, or do they only occur in a limited geographical area? This question is addressed in **Chapter 2**, where the results of a survey are given of the occurrence of ineffective *Frankia* soil populations in various wet *A. glutinosa* sites. Furthermore, additional evidence for the hypothesis that these *Frankia* strains are restricted to permanently inundated ecosystems is presented here.

2) Is the resistance, shown by the host plant *A. glutinosa* to nodulation by ineffective *Frankia* influencing the ineffective *Frankia* population, and are ineffective *Frankia* strains solely dependent on the formation of ineffective nodules on *A. glutinosa* for the maintenance of their soil population. **Chapter 3** addresses some of the problems of obtaining clonal *A. glutinosa* plants for the cultivation of ineffective *Frankia* strains, and for the study of host resistance of alder trees. Also, nodulation resistance in natural alder populations with or without an ineffective *Frankia* soil population was analysed.
3) What is the phylogenetic relationship of ineffective *Frankia* strains from wet *A. glutinosa* soils with Fix^- *Frankia* isolates, and with local effective *Frankia* strains? **Chapter 4** contains an introduction to the use of phylogeny in ecological studies, and a synopsis on phylogenetic tree construction. Data on the phylogenetic position of uncultured ineffective *Frankia* strains with respect to local effective *Frankia* strains, and with respect to Fix^- *Frankia* isolates are discussed, also comparing several different phylogenetic tree-making methods.

Chapter 2

PRESENCE OF INEFFECTIVE NODULE-FORMING *FRANKIA* PARTICLES IN WET ALDER STANDS

2a: Ineffective *Frankia* strains in wet stands of *Alnus glutinosa* L. Gaertn. in the Netherlands

D. J. Wolters, A. D. L. Akkermans and C. van Dijk

Summary: Nodulation experiments using *Alnus glutinosa* L. Gaertn. (Black Alder) seedlings as hosts and soil suspensions as inoculates were conducted to determine the presence and relative amounts of ineffective (non-nitrogen fixing but infectious) *Frankia* strains in 10 water-logged soils of natural alder stands in the Netherlands. Ineffective nodules were formed with soil from six locations, while effective nodules were found at all sites. From three of these locations the majority of the nodules formed were of the ineffective type. These results suggested that ineffective strains form an important fraction of the *Frankia* population of wet soils under black alder vegetation. No correlation was found between the distribution of these *Frankia* strains and single soil chemical components.

Soil Biology and Biochemistry (1997) **29**, 1707-1712 [*)]

INTRODUCTION

The intimate symbiotic relationship between the actinomycete *Frankia* and host plant *Alnus glutinosa* L. Gaertn. (Black Alder) has been documented extensively (Akkermans and Van Dijk 1981, Benson and Silvester 1993). As a rule, all *A. glutinosa* specimen in the field have root nodules (Bond 1977). Nitrogen fixation takes place in these nodules, which allows the plant to grow under nitrogen-poor soil conditions.

The formation of root nodules, ineffective in nitrogen fixation, has been described as a result of nodulation tests with different combinations of *Frankia* strains and host plant species. In some cases effectivity or ineffectivity depended on the compatibility of these *Frankia*-host plant combinations (VandenBosch and Torrey 1983, Van Dijk *et al.* 1988, Kurdali *et al.* 1989). The term used for this type of interaction is facultative ineffectivity. Similar facultative ineffective interactions have been described for legume-*Rhizobium* interactions (Hagedorn 1978, Shantharam *et al.* 1987).

Among the symbiotic *Frankia*, true, or obligate ineffective strains are unable to fix nitrogen in association with any known host plant (Fix^-). This type of *Frankia* strain was first encountered when efforts to obtain *Frankia* strains in pure culture from nodules, that were effective in nitrogen fixation, resulted in *Frankia* isolates, deficient in nitrogen fixing ability (Baker *et al.* 1980, Hahn *et al.* 1988, Lechevalier *et al.* 1983).

Field data on ineffective (Fix^-) *Frankia* are scarce. Only in one case the presence of Fix^- *Frankia* in the soil of three wet alder sites in the Netherlands (Quackjeswater, Meertje de Waal and Voorne's Duin, Valley 7) has been reported (Van Dijk and Sluimer-Stolk 1990). At Voorne's Duin, Valley 7 (VD7), the nodulation capacity (NC) of the soil for ineffective *Frankia* was found to be associated with water-logging of the soil, whereas nodulation capacity by effective *Frankia* remained unaffected. From ineffective nodules resulting from inoculation tests with soil from Voorne's Duin, Valley 7, an ineffective *Frankia* strain, named AgI5, was obtained (A. D. L. Akkermans, unpublished data).

rDNA sequence analyses of some ineffective strains indicated a common ancestry, related to but different from effective *Frankia* strains (Hahn *et al.* 1989a, Hönerlage *et al.* 1994, Mirza *et al.*

1992). Also, in a recent study it was established that there are differences in expression of plant genes between effective and ineffective *Frankia* nodules on *A. glutinosa* (Guan *et al.* 1996).

Ineffective *Frankia*, such as strain AgW1 (Hahn *et al.* 1988), only produced nodules on part of the host genotypes. Resistance of *A. glutinosa* to nodulation by ineffective *Frankia* strains, as expressed by the absence of nodules, has also been reported by Van Dijk and Sluimer-Stolk (1990). The latter authors showed that plant resistance to nodulation by soil populations of ineffective *Frankia* strains is a stable trait, with reproducible resistance percentages among half-sib *A. glutinosa* seedlings (Van Dijk and Sluimer 1994). In contrast, resistance of *A. glutinosa* to nodulation by the effective *Frankia* strain type has never been reported. The term strain type was introduced previously (Van Dijk and Sluimer-Stolk 1990) to designate groups of *Frankia* strains which share one or more distinctive characteristics. In this paper, strain types sp(+) and sp(-) refer to effective strains with or without spore formation, respectively. Obligate ineffective strains from *A. glutinosa* stands will be indicated strain type AgI.

We have surveyed the distribution of ineffective *Frankia* strains, nodulating *A. glutinosa*, in natural and long-term undisturbed alder sites in the Netherlands. Only sites with long-term water-logged conditions were selected.

MATERIALS AND METHODS

Description of study areas and sampling sites

All study sites were selected for the presence of a relatively undisturbed, established *A. glutinosa* vegetation with water-logged soil conditions throughout the year. Most of the areas were governed as nature reserves by the Vereniging tot Behoud van Natuurmonumenten in Nederland (the Society of the Preservation of Nature in the Netherlands; NM), Staatsbosbeheer (the National Forest Service in the Netherlands; SBB), and by the regional organisation Stichting Het Geldersch Landschap (the Gelderland Trust for Natural Beauty; GL), with a minimum of anthropogenic influences.

The locations, described in Table 1, are presented according to the co-ordinates of the Dutch State Survey Grid (S.S.G.).

Table 1 Description of sampling sites, selected for the presence of natural stands *of Alnus glutinosa* on water-logged soils. Shown are the Dutch State Survey Grid (S.S.G) coordinates, the governing body, the soil type, and the plant species accounting for the main ground cover.

Site	S.S.G coordinates:	Governed by[1)]:	Soil type:	Main understory plant species:
Meertje de Waal (MW)	64.3; 432.9	NM	peat	*Rubus fruticosus*
Boezem van Brakel 1 (BB1)	132.6; 424.0	SBB	river clay	*Mentha aquatica*
Boezem van Brakel 2 (BB2)	132.6; 423.6	SBB	sandy loam	*Phragmites australis*
De Wieden (Wie)	199.7; 522.6	NM	peat	*Mentha aquatica*
De Burgvallen (BV)	240.6; 563.4	SBB	sand	*Juncus effusus*
Zeegsersteeg (Zee)	239.0; 565.9	SBB	organic, sand	*Rubus fruticosus*
De Hel (Hel)	168.3; 477.1	SBB	peat	*Phragmites australis*
De Bijleveld (BY)	126.9; 457.7	SBB	sandy loam	*Mentha aquatica*
Staverden 1 (Sta1)	179.7; 477.1	GL	organic, sand	*Scirpus* sp.
Staverden 2 (Sta2)	179.8; 474.6	GL	organic, sand	*Mentha aquatica*

[1)] For full names see text.

Cultivation of test plants

A seed-lot of *A. glutinosa* collected from one tree at Voorne (the Netherlands) was used for the hydroculture of test plants. Seeds were surface sterilised with a sodium hypochlorite solution (1% active chlorine) for 10 min. and left to germinate on 3 mm glass beads in demineralised water. After 2 weeks, seedlings were transferred to 5 l jars containing a half-strength modified Hoagland solution (Quispel 1954a), supplemented with trace elements (Allen and Arnon 1955) and Fe-citrate (0.02 mM). At 6 weeks the seedlings were transferred to full-strength modified Hoagland solution with reduced N-content (0.375 mM NO_3^- as sole source of N). Plants were raised in a growth-chamber at 23°C and 70% relative humidity, and illuminated for 16 h day^{-1} with Philips TL 33 (185 mE s^{-1} m^{-2}, at plant level). After selection for equal size, the plants (48 plants per pot, 2 pots per soil sample) were inoculated and transferred to a greenhouse at 23 ± 2°C. Extra illumination with Philips HPI-T (min. 195 mE s^{-1} m^{-2}, at plant level) was provided for a photoperiod of 16 h day^{-1}. To avoid external contamination with soil-borne *Frankia* the plants were placed under plastic shelters and a strict hygienic regime was maintained for the duration of the experiment.

Collection of soil samples and preparation of inoculates

Soil samples were collected in the last week of November, 1992. From an area of ca. 100 m^2 at each sampling site, 15-20 cores of 5 cm dia. were collected at random from the top 20 cm of the soil. The cores were pooled into plastic bags, and stored at 4°C. Sampling tools were disinfected before sampling at a different site. Prior to inoculation each soil sample was manually mixed. Coarse root fragments were removed. Of the mixed samples 400 ml of soil was suspended into 1.5 l of modified full strength Hoagland solution with reduced N-content. The soil mixture was stirred mechanically for 30 min. at 200 rev. min^{-1}, after which the mixture was sieved through 2 mm and then through 1 mm aseptic filters.

Inoculation of test plants

One day before inoculation the plants were transferred to fresh Hoagland solution with reduced N-content, with the addition of 80 mg ml^{-1} of Previcur® N (Schering AG, Germany), according to Van Dijk and Sluimer (1994). Once a week, the Hoagland nutrient solution and fungicide were replaced. Inoculation was carried out with freshly prepared soil-inoculates. These were added to the plant root systems at the equivalent of 15.8 g of fresh soil from MW, BB1, Wie, Zee, Hel, BV and Sta1 per jar and 20 g of fresh soil from BB2, BW and Sta2, in 4.8 l of nutrient solution per jar.

Assessment of plant nodulation

Six weeks after inoculation the nodules were counted and the nodule types (sp(-), sp(+) or ineffective) identified by examination of Fabil stained fresh sections (Van Dijk and Merkus 1976) with a light microscope. All nodules smaller than 2-3 mm were examined, whereas only 1 - 2 larger nodules were assayed per plant. From each nodule, at least three sections were examined. The presence of at least one mature vesicle cluster in any of the cells infected with *Frankia* characterised effective nodules. Only nodules in which mature vesicle clusters were totally absent were considered to be ineffective. Similarly, sp(-) nodules were typed by a complete lack of sporangia; nodules with at least one mature sporangium were considered to be of the sp(+) strain type.

The nodulation capacity NC (in nodule forming units (nfu's) g^{-1} of fresh soil) for ineffective *Frankia* of the various soil samples was calculated using the formula:

$$NC = Nod(i) / (S \times W)$$

Nod.(i) is the number of ineffective root nodules formed per soil sample. The occurrence of resistant seedlings was quantified by factor S, representing the fraction of test plants, susceptible to ineffective nodulation. W is the total amount of soil (g fresh weight) added to the jar(s) with test plants. For calculating the nodulation capacities for effective *Frankia* in the same soil samples, S = 1 (all plants susceptible).

Chemical analysis of soil samples

The soil samples were analysed, using standard procedures (Troelstra *et al.* 1990), for content of soil moisture, total organic matter, N and P, and for pH (H_2O), pH (KCl), soil conductivity, and concentrations of the ions Cl^-, Ca^{2+}, Mg^+, Na^+ and K^+, For the pH and soil conductivity measurements, the ratio of soil : liquid had to be adjusted from the usual 1:2 to 1:5 for the soil samples Hel, MW and Wie because of the high organic matter content of these soils.

RESULTS

Chemical analysis of soil samples

The physical and chemical soil parameters of the sampling sites (Table 2) showed large differences in organic matter content, ranging from 4 - 87% of the soil dry weight. The mineral components were composed of fractions of sand and/or clay particles. All soils were selected for water-saturated conditions, but soil water content (on dry weight basis) varied between 0.5 and 7 kg kg^{-1} and highly correlated with the organic matter content ($R^2 = 0.99$, with $p < 0.005$). Differences in N and P content are also highly correlated with the soil organic matter content (resp. $R^2 = 0.99$ and 0.61, with $p < 0.05$). The same held true for the chloride and cation levels ($0.51 < R^2 < 0.81$, with $p < 0.05$). Extreme cation and Cl^- levels were found at location MW, which is most likely related to seepage of sea water into the area. The soil pH(H_2O) values ranged from 5.0 to 7.6.

Table 2 Chemical and physical parameters of 20 cm top soil from natural stands of *Alnus glutinosa*.

Site	Soil H_2O ($kg\ kg^{-1}$)	pH (H_2O)	pH (KCl)	% Org. matter (of dry weight)	total N ($mg\ 100g^{-1}$)	total P ($mg\ 100g^{-1}$)
MW	7.40	5.5	5.1	87	3047	83.1
BB1	1.08	6.7	6.1	12	551	62.7
BB2	0.65	7.4	7.0	4	109	19.7
Wie	6.11	5.6	5.1	74	2864	123.2
BV	1.38	5.0	4.6	11	467	71.5
Zee	2.35	5.0	4.6	25	1034	47.7
Hel	3.52	5.5	5.0	33	1215	50.8
BY	0.49	7.6	6.9	4	155	37.7
Sta1	2.46	5.4	4.9	28	934	61.0
Sta2	0.97	5.1	4.4	8	257	43.5

Site	Conductivity ($\mu S\ cm^{-1}$)	Cl^- (mmol $100\ g^{-1}$)	Ca^{2+} (cmol charge kg^{-1})	Mg^{2+} (cmol charge kg^{-1})	Na^+ (cmol charge kg^{-1})	K^+ (cmol charge kg^{-1})
MW	2746	13.9	49.8	22.2	19.6	1.08
BB1	172	0.11	34.6	3.19	0.36	0.29
BB2	187	0.02	12.6	0.85	0.07	0.10
Wie	921	2.73	55.2	9.92	6.14	0.38
BV	346	0.05	9.60	0.57	0.28	0.07
Zee	516	0.41	16.0	2.75	0.55	0.32
Hel	296	0.31	27.6	2.05	0.67	0.25
BY	201	0.07	41.1	1.17	0.21	0.13
Sta1	286	0.14	20.6	1.06	0.32	0.12
Sta2	97	0	1.56	0.24	0.25	0.12

Nodulation capacity of the soil samples

Table 3 presents the pooled nodulation data of two pots per sampling site. Individual treatment of replicate pots was omitted because of a marked effect of random distribution of large numbers of AgI resistant plants among the replicate pots.

There are marked differences in both the numbers and the type of nodules formed from different soil samples. Soil samples from BB2, Wie, BV and Sta2 caused only effective (AgEff) nodules to emerge on test plants, whereas those from MW, BB1, Zee, Hel, BY, and Sta1 also gave rise to ineffective (AgI) root nodules, indicating uneven distribution of effective and ineffective *Frankia* strain types among the sampling sites. In jars with the highest numbers of ineffective nodules (BB1, Zee, and Hel) the occurrence of only 19 - 20% of plants with ineffective nodules pointed to a high share of resistant test plants. As it was expected that the distribution of

nodules among test plants follows a Poisson distribution (Quispel 1954b), non-nodulated plants are not likely ($p < 0.05$) to occur at random at mean numbers of ≥17 or ≥10 for site Hel and BB1, respectively.

Hence, a resistance percentage of 80% for test plant seed-lot Voorne was calculated from equal ratios nodulated and non-nodulated test plants from sites Hel, BB1 and Zee ($Chi^2 = 0.045$ with $p = 0.978$) . The nodulation capacities for ineffective *Frankia* presented in Table 3 were calculated using S = 0.2. For effective nodules all plants (N) were supposed to be susceptible, using S= 1.0 for calculation of the NC. Figure 3 displays the nodulation capacities from test sites in the Netherlands. The site Oostvoorne, Valley 7 (not shown), where this strain type was originally found, is situated close to site MW.

There was no correlation between the nodulation capacities of the soil samples for effective and ineffective *Frankia* ($R^2 = 0.10$, $p < 0.05$). Ineffective nodules were present together with sp(+) (at sites Zee and Sta1) or sp(-) (at sites MW, BB1, Hel and BY) strain type effective nodules. The data also suggest that ineffective *Frankia* had a more limited distribution than effective *Frankia*.

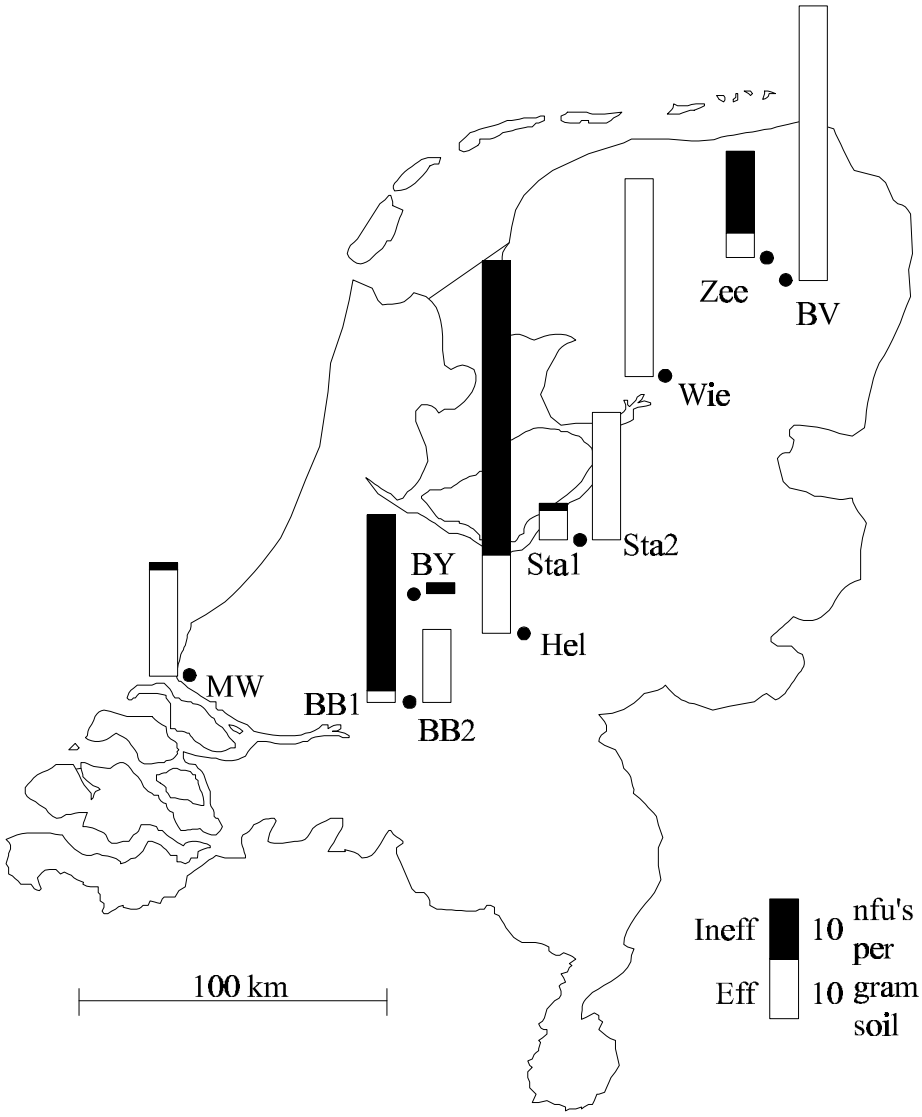


Figure 3 Schematic map of the Netherlands with sampling sites Meertje de Waal (MW), Boezem van Brakel (BB1 and BB2), de Wieden (Wie), de Burgvallen (BV), Zeegsersteeg (Zee), De Hel (Hel), de Bijleveld (BY) and Staverden (Sta1 and Sta2).
Stacked bars represent nodulation capacities of effective (Eff.) and ineffective (Ineff.) nodules in nodule forming units g^{-1} fresh soil (See Table 3). Bar sizes in the legend represent 10 nfu's per gram of soil.

The nodulation data did not correlate significantly with any of the soil chemical data discussed above ($0.04 < R^2 < 0.29$, with $0.11 < p < 0.96$). Also, there were no indications that the presence of ineffective *Frankia* was associated with the dominant understory species.

Microscopic observation of cross-sections of ineffective nodules showed a pattern similar to that described by Van Dijk and Sluimer-Stolk (1990). No marked differences in appearance of the ineffective *Frankia* nodules between the different soil samples were found, neither in microscopic sections, nor in morphology.

Table 3 Nodulation response of *Alnus glutinosa* test plants to inoculation with soil samples from the various areas. Data on nodulation were collected 6 weeks after inoculation. Data represent the total number of ineffective nodules present on n plants; total number of effective nodules and presence or absence of sporangia; total number N of test plants after 6 weeks; nodulation capacities of ineffective (AgI) and effective (AgEff) *Frankia* (nodule forming units g^{-1} fresh soil).

Site	Ineffective nodulation		Effective nodulation			Nod. Cap.	
	number of nodules	number of plants (n)	number of nodules	number of plants (N)	nodule type	AgI	AgEff
MW	9	3	580	92	sp(-)	1	18
BB1	185	18	63	95	sp(-)	29	2
BB2	0	0	484	89	sp(-)	0	12
Wie	0	0	1051	95	sp(+)	0	33
BV	0	0	1838	94	sp(-)	0	46
Zee	88	19	133	95	sp(+)	14	4
Hel	307	18	396	95	sp(-)	49	13
BY	13	9	2	96	sp(-)	2	<0.1
Sta1	6	5	155	95	sp(+)	1	5
Sta2	0	0	848	95	sp(+)	0	21

DISCUSSION

Ineffective *Frankia*, similar to strain type AgI from the Isle of Voorne (Van Dijk and Sluimer-Stolk 1990) were detected over a wide range of physico-chemical soil conditions under wet alder stands, located at different geographical regions in the Netherlands. The observations confirmed that strain type AgI is a common and significant part of *Frankia* communities in wetland ecosystems with natural stands of *A. glutinosa*. As sites with drier soils were not included in this study, the importance of waterlogged soil conditions for maintenance of ineffective populations (Van Dijk and Sluimer-Stolk 1990) remains to be proven.

The plant trapping method allowed qualitative detection of nodule forming units of *Frankia* strain types and estimation of the relative abundance among samples. Interpretation of nodulation capacities is limited by the detection level of the plant trapping method, leaving room for

ecological significance of populations with zero nodulation capacities. Also, the biotic and abiotic composition of different soils may interfere with nodule development and comparability of nodulation capacities. At sites BV, Wie and Sta2 with zero nodulation capacities for strain type AgI, effective *Frankia*, that were present at high nodulation capacities, may have limited the nodule development by strain type AgI (Van Dijk and Sluimer-Stolk 1990). The use of dilution series of soil suspensions in the plant trapping method was beyond the scope of this survey, but this may have added to underestimation of high nodulation capacities (Quispel 1954b, Van Dijk 1984).

The nodulation capacities for the AgI strain type of the areas in this study were similar to the number of nodules found by Van Dijk and Sluimer-Stolk (1990) at locations Quackjeswater and Meertje de Waal, but much lower than the nodulation capacity of about 700 nfu's g^{-1} soil found for Voorne's Duin, Valley 7. The latter site shared numerical dominance of the ineffective strain type with sites BB1, Zee and Hel of this survey. Effective nodulation capacity was within the range previously reported for sp(-) alder sites, although at the lower end of the spectrum (Van Dijk 1984). Only the nodulation capacity of area BY for effective *Frankia* can be considered to be far below values normally found for wet alder stands.

The resistance percentage of seed-lot Voorne in response to ineffective *Frankia* sources BB1, Zee and Hel in this study showed close similarity with other seed-lots from that area in response to strain type AgI- Voorne in former studies (Van Dijk and Sluimer 1994). This indicated similarity among geographically separated populations of *Frankia* strain type AgI concerning genetic interactions with specific genotypes of *A. glutinosa*.

Resistance or susceptibility of host trees at the study sites might influence the population size of strain type AgI and hence contribute to a scattered pattern of nodulation capacities as compared with effective *Frankia* strains. Such dependence of ineffective *Frankia* populations on susceptible hosts might explain the absence of nodulation from the soil of three study sites. The resistance of seed-lots and individual trees at sites with and without ineffective *Frankia* is presently under study.

2b: Evidence for a decline of ineffective *Frankia* in the soil of a wet alder dune valley

Summary: Ineffective *Frankia* have been encountered in a wet dune valley, where they appeared to be constricted to waterlogged areas at this location. Since this early report on the occurrence of an ineffective *Frankia* soil population, the sampling site has been gradually drying out due to a lowering of the soil water-table. Nodulation experiments have demonstrated that at the same time, the nodulation capacity of the soil for ineffective *Frankia* has diminished considerably, and during the last survey, ineffective *Frankia* could only be detected in soil from the sole wet sampling site at this location. This provides strong, be it circumstantial, evidence that ineffective *Frankia* is dependent on soil waterlogging for maintenance of an active soil population.

INTRODUCTION

As described in chapter 2a, ineffective *Frankia* form a common, and a sometimes relatively numerous microbial soil component of wet alder locations. Van Dijk and Sluimer-Stolk (1990) were the first to report on this particular *Frankia* strain type in natural stands of *Alnus glutinosa*, and they discovered a connection between ineffective *Frankia* nodulation potential of the soil, and the water-logged conditions of the sampling site. In the previous section of this chapter we reported that ineffective *Frankia* strains also occur in other wet alder stands in the Netherlands. A direct correlation with soil humidity could not be made because dryer alder locations were not sampled.

Although confirmation of ineffective *Frankia* dependency on waterlogged soil conditions may only come with the isolation and physiological characterisation of pure cultures of these *Frankia* strains, some additional, be it circumstantial, evidence can be obtained from nodulation experiments. In this section, changes in the nodulation capacity of the soil of Voorne's Duin, Valley 7 over a number of years are presented.

MATERIALS AND METHODS

Study area and sampling sites

Location Voorne's Duin, Valley 7 (VD7; State Survey Grid 64.1; 436.8) was surveyed in three different years for the presence of ineffective *Frankia* in the soil.

Van Dijk and Sluimer-Stolk (1990) collected soil samples in 1982. They described VD7 as part of a 60-years-old primary dune valley covered with a dense vegetation of woody species and herbs. At location VD7, two main vegetation types could be found. Both run parallel to the path indicated in Figure 4. The first, and highest consisted of a mixture of *Betula pendula* and *Alnus glutinosa*, with a dense understory of *Mentha aquatica*, *Cardamine pratensis*, *Lycopus europaeus*, *Hydrocotyle vulgaris*, and *Iris pseudacorus*. In this zone, sampling site VD7-4 was chosen. It was waterlogged in winter and early spring, but in summer the upper 20 to 30 cm of the soil were well drained. Further away from the path, and at a lower elevation was a strip of *A. glutinosa*, with a sparse understory of *M. aquatica*. Within this zone, sampling sites VD7-1 and

VD7-2 were situated. These sites were inundated each year for 8-10 months, and had an almost permanently waterlogged soil. Sampling site VD7-3 was intermediate between the other two sites in position, humidity, and understory composition.

In 1992, a second survey at this location was conducted. Three sampling sites were chosen, with VD7-1 (’92) corresponding with the earlier site VD7-1 (’82), and VD7-2 (’92) corresponding with site VD7-2 (’82). The third site, VD7-5 (’92), was situated beyond (and below) the *A. glutinosa* zone, where a *Salix* sp. swamp was situated. Vegetation cover and composition were unchanged, but due to a succession of dry winters, the soil water-table had dropped considerably. Only VD7-5 (’92) was almost permanently inundated, site VD7-1 (’92) was, while still water-saturated, no longer inundated outside the winter months and early spring. Site VD7-2 (’92) was also much dryer.

Location VD7 was revisited early in 1995, and the soil water-table had again dropped considerably, although the vegetation cover had not changed perceptibly over the past four years. Sites VD7-1, 6 and 7 were chosen, with site VD7-1 (’95) at the approximate location of previous sites VD7-1 (’82) and VD7-1 (’92). Sampling sites VD7-6 (’95) and VD7-7 (’95) were situated SSW and NNE of site VD7-1 (’95), respectively. Only at site VD7-7 (’95) the soil was water-saturated, as it was located next to a wide ditch which had been dredged about four years earlier.

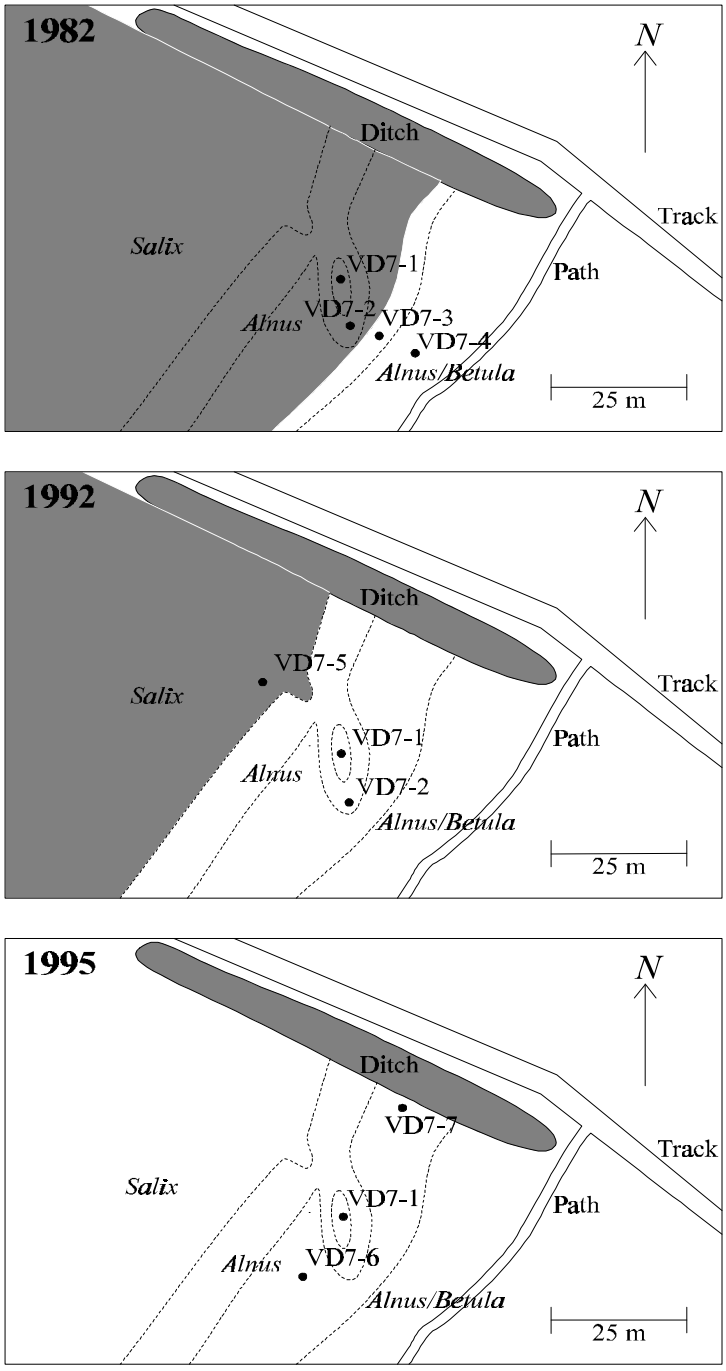


Figure 4 Map of location Voorne's Duin, Valley 7 (VD7), over three years (1982, 1992 and 1995). Waterlogged soil is indicated by the grey areas, while sampling sites are marked with dots. The dotted lines depict soil elevation (approx. 10 cm per elevation line). Dominant tree species are also shown in the map, as mixed *Alnus*/*Betula*, *Alnus*, and *Salix* vegetations.

Nodulation experiment

Van Dijk and Sluimer-Stolk (1990) used a seed-lot from Voorne, and for the second nodulation experiment, Voorne seed-lots were used as well. For the 1995 nodulation experiment, a seed-lot from a different geographical location was used (Location De Wieden; See Fig. 3, p. 40). Although the resistance percentage (see chapter 3b) against ineffective *Frankia* nodulation of the Voorne seed-lots was much higher than that of the De Wieden seed-lot (± 80% resistance versus ± 50% resistance) this was corrected for while calculating the nodulation data, as was described in chapter 2a.

Seedling cultivation and inoculation occurred as described previously. Numbers of seedlings were generally about 36 to 48.

The nodulation capacity NC (in nodule forming units (nfu's) g^{-1} of fresh soil) for ineffective *Frankia* of the various soil samples was calculated using the known fractions of test plants per seed-lot, susceptible to ineffective nodulation. To be able to compare all nodulation data, the numbers of ineffective nodules Van Dijk and Sluimer-Stolk (1990) obtained from inoculating pots with 20 g of soil were taken.

RESULTS

Nodulation capacity

Table 4 presents the nodulation capacities calculated from the numbers of ineffective *Frankia* strains found on the root systems of the test plants. A number of observations can be made from these data. Firstly, in 1982, the nodulation capacity of the soil from sampling sites VD7-1 and VD7-2 for ineffective *Frankia* was highest, with 86-116 nodules formed per g of soil. Zero nodulation was encountered in the well-aerated soil of site VD7-4, and at VD7-3 the intermediate value of 39 nodules per g of soil was found. In 1992, overall soil nodulation capacities were clearly lower, with again site VD7-1 in the *Alnus*-zone showing the highest value. It is striking that soil from site VD7-5 in the *Salix* vegetation-zone produced an almost equally high nodulation capacity. Again, the slightly less wet soil at site VD7-2 had a lower nodulation capacity. Finally, in 1995, the only site with a detectable ineffective *Frankia* soil population was VD7-7, near the ditch. Soil from site VD7-1 did no longer form any ineffective *Frankia* nodules on the test plants.

Table 4 Ineffective *Frankia* nodulation capacity of soil from Voorne's Duin, Valley 7 in 1982, 1992 and 1995.

	Nodulation Capacity						
Year	Location with nodulation capacity (nodules g^{-1} soil)						
	VD7-1	VD7-2	VD7-3	VD7-4	VD7-5	VD7-6	VD7-7
1982 [1]	86.3	116.2	39.0	0			
1992 [2]	13.4	7.2			12.2		
1995 [2]	0					0	18.3

[1] Van Dijk and Sluimer-Stolk (1990)
[2] This study

DISCUSSION

The data presented in Table 4 clearly showed that the soil nodulation capacity for ineffective *Frankia* at location VD7 has declined over the years. Interestingly, this process seems to have been proceeding in concordance with the decrease of soil water-content. In 1982, most of location VD7 still was water-logged and/or water-saturated for the greater part of the year. In 1992, only the *Salix* vegetation-zone and the ditch were permanently water-saturated and waterlogged in winter, while the *Alnus*-zone was only water-saturated, but not water-logged in winter. In 1995 the whole of location VD7 was only slightly moist in winter (water-table at -15-30 cm). The only site with water-saturated soil bordered the ditch at the north-western edge of VD7. This site harboured the only detectable ineffective *Frankia* soil population in 1995.

It should be noted that the absolute quantities of nodulating capacities presented in Table 4 are only approximations of the true NC values, which would be obtained when using a soil dilution series. Furthermore, only limited numbers of test plants were used for the nodulation experiments. Because of this, the nodulation data must be primarily considered in a qualitative fashion.

Even so, the data strongly supports the hypothesis that the ineffective *Frankia* soil populations (at location VD7) are dependent on water-saturation of the soil.

Chapter 3

RESISTANCE OF NATURAL *ALNUS GLUTINOSA* TREES TO NODULATION BY INEFFECTIVE *FRANKIA* AND ITS POSSIBLE ECOLOGICAL IMPLICATIONS

3a: Micropropagation of field- and greenhouse grown *Alnus glutinosa* plants

INTRODUCTION

It has been shown by Van Dijk and Sluimer (1994) that some *A. glutinosa* seedlings remain free of nodules, while others are nodulated profusely when inoculated with soil containing ineffective *Frankia* particles. Of some half-sib seed-lots, up to 90% of the plants were found to be resistant to nodulation by ineffective *Frankia*. It has been demonstrated experimentally that both compatibility with ineffective *Frankia* (i.e. the ability to form nodules) and the nodulation speed are genetically determined by the host. For more efficient research of ineffective *Frankia* nodulating *A. glutinosa*, multiplication of certain susceptible alder individuals through cloning was considered to be very useful (Hahn *et al.* 1988), and micropropagation is virtually essential for the study of resistance patterns among natural *A. glutinosa* populations. Although various methods for micro-propagation of *Alnus* spp. have been described (Séguin and Lalonde 1990), most of these are quite complicated, and might not work on field-grown, mature alder plants. In the present chapter, several methods for the multiplication of *Alnus glutinosa* were tested, and their usefulness for nodulation studies was investigated.

MATERIALS AND METHODS

A) Two-step multiplication (Périnet and Lalonde 1983)

For the production of a large number of identical alder clones for biotest purposes, a two-step tissue culture technique was selected. This technique was specially designed to increase the number of propagates obtained from each target plant (Périnet and Lalonde 1983). Plant cuttings (explants) of 2 cm with at least one lateral bud were surface-sterilised using 0.42% NaOCl, and were placed in a ‘multiplication medium’ containing the plant hormone 6-benzylaminopurine (BAP). This resulted in excessive shoot formation on each of the explants. The newly formed shoots were separated from the original explants and were placed in ‘rooting medium’ containing indolebutyric acid (IBA), which induces root formation. The basal salt medium that was used has been described by Murashige and Skoog (1962), and was supplemented with 3% (w/v) of sucrose, and

brought at a final pH of 5.5. Alder shoots, both from greenhouse- and field origins, were surface-sterilised using the method described by Jones *et al.* (1977). This method was successfully used by Tremblay and Lalonde (1984) and Tremblay *et al.* (1984) for the micro-propagation of various *Alnus* spp. Instead of using the 'multiplication agar medium' with sucrose, explants were also placed on liquid basal medium without sucrose (on sterile glass beads). Newly formed shoots were placed on (likewise sucrose-free) rooting medium.

B) Stem cuttings (Van Dijk and Sluimer 1994)

Van Dijk and Sluimer (1994) have described the standard method that was used for cloning greenhouse-grown alder plants. Stem sections, 4 cm long with at least one lateral bud, were wiped with 70% (v/v) ethanol, rinsed in sterile demineralised water, and placed on perlite saturated with demineralised water. Trays with cuttings were placed in growth cabinets (Conviron E15) at 25°C and 80% relative humidity, with a 14-h. photoperiod. Various modifications in rooting medium (peat/sand mixtures, peat/perlite mixtures), application of soil sterilisation, and the use of fungicide (1% Captan) and auxin (indole acetic acid or indole butyric acid) were also tested.

C) Tissue culture (Van der Tang 1995)

A tissue-culture method (Van der Tang 1995) was employed, using buds from actively growing alder plants, and dormant "winter" buds. For the sterilisation, a two-step incubation in a sodium hypochlorite solution (2-4% active chlorine, 15-30 min. incubation) with Tween 20 was used, punctuated by a 10 h. period of incubation in malt extract broth with glucose. This incubation period was intended to induce fungal and bacterial sporangia to germinate, and thus to become vulnerable to the second sterilisation step. Buds were placed in glass tubes with glass beads on a liquid 'multiplication medium' (pH 6.5) containing (per l) 4.43 g basal salt medium (Murashige and Skoog 1962), 20 g sucrose, 10 active carbon, 0.25 g anti-oxidant, and 1µM BAP. To further reduce fungal growth, buds were treated with Benlate (Sbay *et al.* 1989) by soaking in a 3 g l^{-1} aqueous solution for 24 h.

D) Root clones (chapter 3b)

Growing shoots were removed from near the base of mature trees together with roots and bark, and transplanted into pots of soil (2 parts of river sand, 1 part of peat) under greenhouse conditions. The occurrence of both roots and shoots near the base of a tree coincided with a frequently changing, but high water table at the sampling site. These explants were called 'root-clones' (chapter 3b). Secondary shoots, formed from the transplants under greenhouse conditions, were subsequently used for cloning, according to method B).

RESULTS AND DISCUSSION

When using the two-step multiplication (A), greenhouse-derived explants remained axenic, but field-derived plant material quickly became overgrown with mainly fungal contaminants. Some of the contaminants were identified at genus-level as *Acremonium* spp., *Penicillium* sp., *Alternaria* sp. and several *Trichosporon* spp, members of which are known to be common soil saprophytes. Furthermore, several yeasts and bacteria were found. The main cause of this microbial bloom was probably the high sucrose-level in the multiplication medium. Increasing the strength of the sterilising solution (from 0.42% w/v NaOCl up to 4 %) did not significantly decrease the infections, but only postponed them. Only when explants were placed on basal medium without sucrose (on glass beads), survivability increased. Of the newly formed shoots that were placed on (likewise sucrose-free) rooting medium, a small proportion formed roots, but field-derived plant material never survived.

The method of cloning greenhouse-grown alder plants using stem cuttings (B) was quite straightforward, with survival rates commonly exceeding 50%. The success rate appeared to be determined by the host plant genotype: cuttings from some plants consistently withered and died, while others rooted quickly without many casualties. Stem cuttings from field-grown alder plants, however, suffered a mortality rate of over 99%, which was mostly caused by fungal infections. Changing the rooting material, application of soil sterilisation, the use of fungicide, and/or auxin did not improve the survival rate. In fact, the original method

(Van Dijk and Sluimer 1994) of only wiping stem cuttings with 70% ethanol, and placing them on water-saturated perlite, was superior to any of the implemented changes.

The tissue-culture method (C), using buds from actively growing alder plants, showed that it was possible to obtain "axenic", viable *A. glutinosa* tissue cultures. Even so, the best achievement was a mere 14% survival. Winter buds appeared to be the most suitable sterilisation subjects, because these are closed tightly, and were unlikely to get damaged by the surface-sterilisation. The use of Benlate (Sbay *et al.* 1989) as a fungicide did not significantly improve viability and survival of the explants.

While some rooted clones were directly obtained as cuttings from field material using method B, most of the alder material used for cloning was taken from stem cuttings of so-called 'root-clones' (D). Mortality of the transplants was low, but some took several months to come out of hibernation. The secondary shoots rooted much better than field material, although the mortality rate of approximately 75% was still too high. Major bottlenecks were finding suitable starting material from each prospective tree, and the long time needed for the two-step cloning process. Still, this method for obtaining clones from alder field material was better than any of the methods described above, and the majority of the rooted clones from alder trees in natural populations were obtained in this way.

A summarising survey of the advantages and disadvantages of the four cloning methods is given in Table 5. It can be concluded that the root cloning was the best way to propagate field-grown *A. glutinosa*, although this method also has several drawbacks.

Table 5 Summary of micropropagation methods used to obtain rooted cuttings of *A. glutinosa*, the approximate success rates, and the advantages and disadvantages of each method.

Method of micro-propagation	Explant survival (%)		Advantages	Disadvantages
	Greenhouse material	Field material		
A) 2 step multiplication	0% survival	0% survival	Many clones from every mother plant.	High sucrose level greatly increases chances for infection.
B) Stem cuttings (non-axenic)	10-60% survival	>0,1% survival	Technically simple; low work load, consistent	Only few clones per mother plant; low survival of field grown plants
C) Tissue culture	14% survival	Not determined	Possibility of obtaining axenic plant material.	Difficult; lengthy process; success rate too low (even without rooting)
D) Root clones	Not determined	25%	Good chances of producing rooted clones.	Lengthy process; suitable cloning material hard to find.

3b: Ineffective *Frankia* and host resistance in natural populations of *Alnus glutinosa* (L.) Gaertn.

D. J. Wolters, C. van Dijk, A. D. L. Akkermans and J. W. Woldendorp

Summary: *Alnus glutinosa* (Black Alder) populations are known to exhibit a variable degree of incompatibility to root nodule formation by ineffective *Frankia*. The relationship between the occurrence of ineffective *Frankia* in wet stands of black alder and the degree of resistance to nodulation by ineffective *Frankia* of seed-lots and clones of alder trees of these particular locations was studied through soil inoculation experiments. The average percentage of resistant plants (R-frequency) among the seed-lots from locations with an ineffective *Frankia* soil population was equal to, or higher than the R-frequencies of locations without ineffective *Frankia*. The mean R-frequency was highest for the seed-lots of the location from which the soil inoculant was taken. These results strongly suggest that ineffective *Frankia* are not strictly dependent on susceptible *A. glutinosa* for the maintenance of their population size. The fungus *Penicillium nodositatum* also nodulated *A. glutinosa* seedlings. Whereas a negative interaction with the ineffective *Frankia* nodulation was found, this did not have a significant effect on the R-frequencies of the seed-lots that were tested, suggesting that the ineffective *Frankia* nodulation adversely affected the myco-nodulation, and not vice versa.

Submitted

INTRODUCTION

Communities of diverse *Frankia* populations in the soils of natural stands of *Alnus glutinosa* have been characterised at the levels of nodule morphology, host specificity and 16S RNA characteristics (Benson and Silvester 1993). A distinct type of *Frankia* from these soils was recognised by the production of ineffective, i.e. non-N_2-fixing nodules in nodulation tests (Hahn *et al.* 1988, Van Dijk and Sluimer-Stolk 1990) and by host-intraspecific differential incompatibility which is so far unique among Frankiae (Van Dijk and Sluimer-Stolk 1990, Van Dijk and Sluimer 1994).

Ineffective *Frankia* have been found in high numbers in the soil of a number of wet alder sites but could not be detected in others (Van Dijk and Sluimer-Stolk 1990, Wolters *et al.* 1997a). Ribosomal DNA sequence analysis of (uncultured) ineffective *Frankia* from various wet alder sites showed that sequences derived from ineffective nodules differed significantly from those of effective ones, but were rather homogenous among themselves (Wolters *et al.* 1997b, see chapter 4). It is not clear why at some sites ineffective *Frankia* were abundant, yet could not be detected at other sites with similar characteristics. An explanation might be found in the distribution of compatible and resistant host genotypes among the study sites, thus regulating the potentials for symbiotic growth of ineffective *Frankia* and hence the sizes of ineffective soil populations.

In the present paper the relationship between the occurrence of ineffective *Frankia* in wet alder stands and the degree of resistance to ineffective *Frankia* nodulation of the alder trees of these particular locations is studied. It is thought that in case of an obligatory dependency of ineffective *Frankia* on the alder tree, a high proportion of susceptible alder individuals would be present. On the other hand, in case of ineffective *Frankia* being independent of growth and multiplication inside susceptible alder trees, no relationship between the occurrence of ineffective *Frankia* particles in the soil and resistance among alder individuals may occur. In the latter case, even a selection for resistant alder individuals would be possible as the ineffective *Frankia* might be considered to be a (minor) pathogen to the alder (Van Dijk and Sluimer 1994). An examination of the relationship between the occurrence of ineffective particles and the degree of resistance of alder trees was carried out through the

inoculation of different seed-lots from three sites with and two sites without an ineffective *Frankia* soil population using a soil suspension containing ineffective particles.

A fungal species has also been known to infect *A. glutinosa* (Van Dijk 1979, Capellano *et al.* 1987), inducing so-called myco-nodules, which morphologically resemble young actinorhizal nodules (Capellano *et al.* 1987). Isolates from these myco-nodules were first (incorrectly) identified as *Penicillium nigricans* (Van Dijk 1984), but similar isolates from France were recognised as *Penicillium nodositatum* nov. spec. (Valla *et al.* 1989, Sequerra *et al.* 1997). As the soil used in our experiments produced significant numbers of these myco-nodules, the interaction between *Frankia* and the *Penicillium* nodule-development was also studied.

MATERIALS AND METHODS

Sampling Sites

Five sites were selected from a previous survey (Wolters *et al.* 1997a) of which three, 'Boezem van Brakel 1' ('BB1'), 'Zeegsersteeg' ('Zee') and 'De Hel' ('Hel') had a numerically significant ineffective *Frankia* soil population, whereas at two sites, 'De Wieden' ('Wie') and 'Burgvallen' *Frankia* could be detected (Fig. 5). All sites are part of nature reserves and had relatively undisturbed, established *A. glutinosa* vegetation with waterlogged soil conditions throughout the year. More detailed information on the sites was given in Wolters *et al.* 1997a (see chapter 2).

Seed collection

At each sampling site, fruit cones were collected separately from four individual *A. glutinosa* trees nearest to the original soil sampling sites (Wolters *et al.* 1997a). Seeds were harvested from the cones and stored in paper bags at room temperature. From each sampling area, 4 (3 in the case of site BV) different half-sib seed-lots were thus obtained (see Fig. 5; from trees at BB1: 1, 5-7; Hel: 2-5; Zee: 1-4; Wie: 1-4; BV: 1-3). Trees numbered otherwise were not used for nodulation experiments, but provided material for cloning purposes, or were screened for the presence of ineffective root nodules.

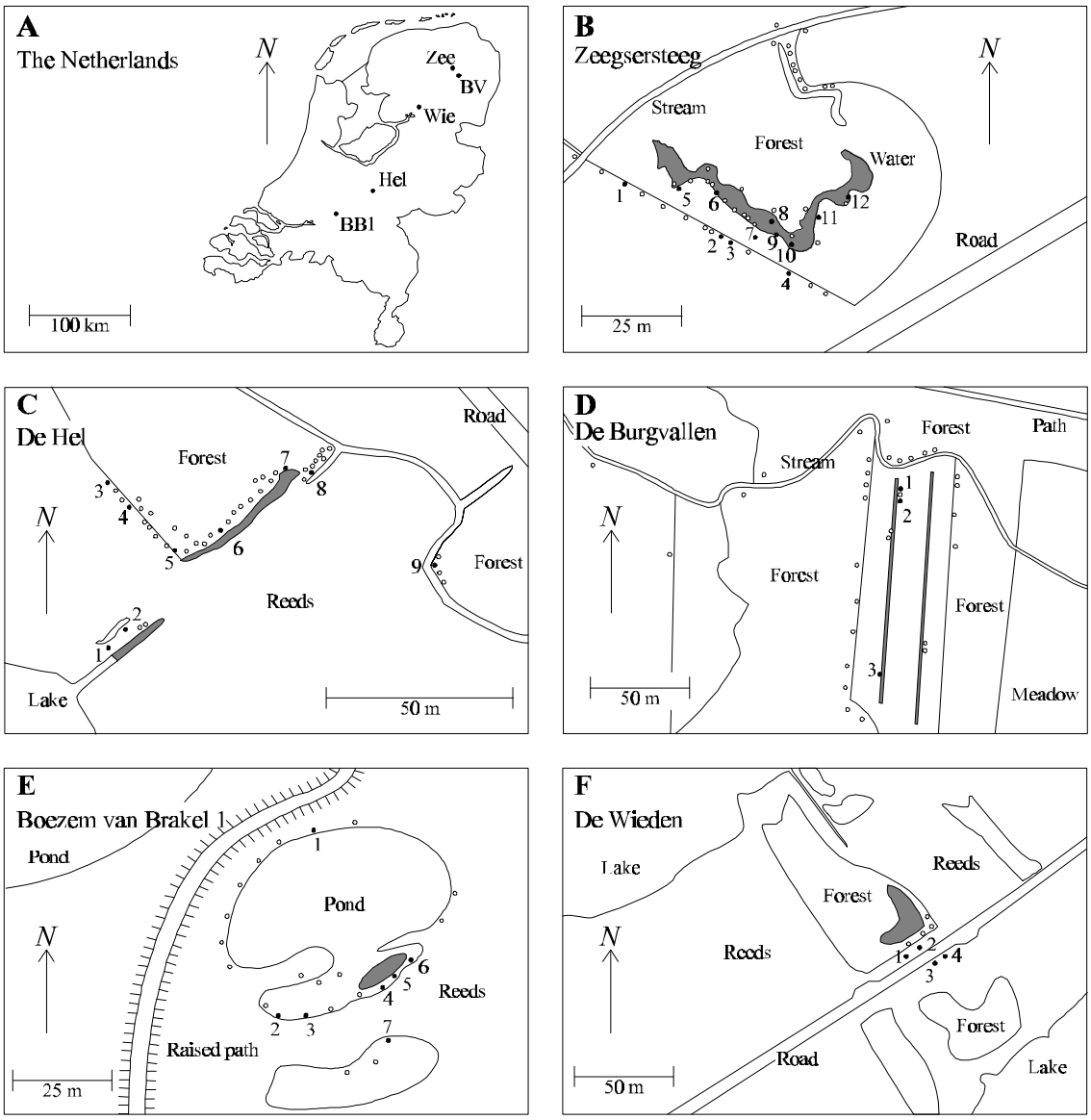


Figure 5 Map of the Netherlands (A) with the positions of locations ‘Zeegsersteeg’ or ‘Zee’ (B), ‘De Hel’ or ‘Hel’ (C), ‘De Burgvallen’ (D) or ‘BV’, ‘Boezem van Brakel 1’ or ‘BB1’ (E), and ‘De Wieden’ or ‘Wie’ (F). The grey areas in the detailed maps show the sites where soil samples were collected, as was described in a previous study (Wolters *et al.* 1997a, see also chapter 2). Ineffective *Frankia* soil populations were present at locations ‘Zee’, ‘Hel’ and ‘BB1’ whereas they were absent at locations ‘BV’ and ‘Wie’. Solid bullets with numbers indicate the *A. glutinosa* trees used for seed sampling and cuttings (this chapter). Open bullets show the general location of some of the unsampled alder trees.

Cultivation of test plants from seeds

Seeds were surface sterilised with a sodium-hypochlorite solution (1% active chlorine) for 10 min. and left to germinate on 3 mm glass beads in demineralised water. After 2 weeks the plants were transferred to 5 litre jars containing a half-strength modified Hoagland solution (Quispel 1954a). Trace elements were added according to Allen and Arnon (1955), and Fe-citrate (0.02 mM) was used as the iron source. At 6 weeks the seedlings were transferred to full-strength modified Hoagland solution with reduced N-content (0.375 mM NO_3^- as sole source of N). Plants were raised in a growth cabinet at 23°C and 70% relative humidity, and illuminated for 16 h day^{-1} with Philips TL 33 (185 mE s^{-1} m^{-2}, at plant level). One day before inoculation the plants were transferred to fresh Hoagland solution with reduced N-content, with the addition of 80 mg l^{-1} of Previcur N (Schering AG, Germany), according to Van Dijk and Sluimer (1994). This treatment was continued by weekly replacement of the Hoagland nutrient and fungicide solutions. Plants were randomly distributed over the jars (initially 48 plants per jar, approx. 96 per seed-lot). Seed-lots were distributed at random among the jars with approx. 8-12 seedlings per jar. For logistic reasons, the total experiment was split up into four sub-experiments. The plants were inoculated with a soil suspension prepared from 'BB1' soil as described below. The jars were transferred to a greenhouse at 23 ± 2°C where extra illumination with Philips HPI-T (≤ 195 mE s^{-1} m^{-2}, at plant level) was provided for a photoperiod of 16 h day^{-1}.

Cultivation of test plants using cloned individuals

While some rooted clones were directly obtained from field material, most of the alder material used for cloning was taken from twig cuttings of so-called 'root-clones', pieces of bark with both roots and buds, separated from the base of mature trees, and transplanted into pots of soil under greenhouse conditions as described above (see chapter 3a). Shoots formed under such conditions were subsequently used for cloning. This procedure was followed, as it appeared that shoots grown in the greenhouse were much easier to clone than field material. Stem sections of ± 5 cm including 1-2 lateral buds were defoliated, and wiped with 70% (v/v) ethanol, before they were placed on Perlite saturated with demineralised water. Containers with cuttings were covered with a sheet of Perspex and placed in a growth cabinet under climatic conditions as described above. Cuttings were inspected once a week for root formation. Rooted cuttings were placed in one-litre

jars, covered with plastic bags, containing a half-strength modified Hoagland solution. When the root systems had adequately developed, the clones were inoculated with 'BB1' soil as described below.

Inoculum preparation

Soil samples were collected from site 'BB1' which had a high ratio of ineffective : effective *Frankia* nodule forming units (Wolters *et al.* 1997a). From an area of ca. 100 m^2, 15-20 cores of 5 cm diameter were collected at random from the top 20 cm of the soil. Before each core was taken, the soil was cleared from litter. The cores were pooled into plastic bags, and kept at 4°C until used. The whole procedure was carried out with the utmost care to prevent contamination by external sources. Prior to inoculation, the soil sample was mixed manually and coarse fragments were removed. Of the mixed samples 400 ml of soil was suspended into 1.5 l of the modified full strength Hoagland solution with reduced N-content. The soil mixture was stirred mechanically for 30 min. at 200-rev. min^{-1}. Particles > 1 mm were removed from the soil homogenate by sieving through 2 and 1 mm filters. For inoculation, freshly prepared soil-inocula were added to the plant root systems, at the equivalent of 20 g of fresh 'BB1' soil l^{-1} of nutrient solution.

Assessment of seed-lot nodulation

Eight to nine weeks after inoculation the plant roots were scanned for nodules. Pilot experiments had shown that after 6-8 weeks, both effective and ineffective *Frankia* nodulation was at its maximum. The nodules were manually cut, stained with Fabil reagent and microscopically examined for the presence of *Frankia* vesicles (Van Dijk and Merkus 1976). Nodules in which no mature vesicle clusters could be detected were considered to be ineffective.

In general, large nodules appeared to contain only effective *Frankia*, but small nodules were found to be induced either by ineffective *Frankia* or by *P. nodositatum* (Van Dijk 1984, Valla *et al.* 1989). The myco-nodules were also counted. Raw data, viz. numbers of nodules (ineffective, effective *Frankia* nodules and myco-nodules) per plant were used to calculate the percentage of seedlings per seed-lot resistant to ineffective *Frankia* nodulation (R-frequency) and also to determine interactions between the different nodule types. R-frequencies per seed-lot were corrected for the estimated number of plants without ineffective nodules that could be ascribed to

Poisson distribution of nodules at low average nodule numbers. These corrections are based on random distribution of nodules among seedlings (Quispel 1954b). Effects of the study sites and of seed-lots on the seed-lot R-frequencies were tested using maximum likelihood analysis with binomial errors (SAS/STAT 6.06, procedure GENMOD; see User's Guide).

Effects of the study sites and of seed-lots on seedling nodulation were examined, applying a similar maximum likelihood analysis with binomial errors. Instead of using absolute numbers of nodules per plant, the GENMOD procedure converts these absolute numbers into shares of effective and ineffective *Frankia* nodules and of myco-nodules.

The absolute numbers of nodules per plant were used to test (for each seed-lot) the distribution of the three types of nodules (effective and ineffective *Frankia* and myco-nodules) over the test plants for Poisson fitting.

Interference between ineffective and effective *Frankia* and myco-nodulation was tested with Chi-square tests (using contingency tables), not only for the total data set, but also for the seed-lots separately. Here, only presence or absence of nodules was taken into account.

Search for ineffective *Frankia* nodules on field-grown alder roots

From 7 individual trees at site 'Zeegsersteeg', (marked in Fig. 5B as trees 5, 6, 7, 8, 9, 10, and 11) the root system was screened for the presence of ineffective root nodules. At least 25% of the surface root system of each tree was harvested. Fine roots (1-2 year old) were surveyed for the presence of small *Frankia* root nodules or nodule primordia using a binocular at 15x magnification. Small nodules (< 1-2 mm) were collected and examined for the presence of ineffective *Frankia* as described above.

RESULTS

Cultivation of plants from seeds

Seedling quality varied widely between seed-lots, as well as plant survival during inoculation. The seed quality of seed-lots BV-2 and BV-3 was quite low, with only a fraction of the seeds germinating. In the case of BV-3 the seed-lot also suffered from a fungal infection which overgrew the seedlings within 2 weeks after the first seeds germinated, in spite of the surface sterilisation of the seeds. For the same reason, a fourth seed-lot was omitted from the experiment. From Table 6, column 2, the number of plants for each seed-lot at the end of the experiment gives an indication of the seedling survival, starting with approximately 100 plantlets prior to inoculation. Especially with seed-lots Hel-3, Hel-5 and Zee-3, plant survival was less than 50%.

Assessment of seed-lot nodulation

Previous experiments with soil from site 'BB1' have shown that the majority of the *Frankia* 'nodule forming units' consisted of the ineffective strain type (Wolters *et al.* 1997a, chapter 2). Contrary to previous observations on this soil, high mean numbers of myco-nodules were observed, in some cases exceeding the mean numbers of *Frankia* nodules (see Table 6). Since fungi and *Frankia* may compete for nodulation sites, both *Frankia* and myco-nodules were included in the analysis of the nodulation data. Whereas myco-nodules were present, no endo- or ecto-mycorrhizal infections were encountered on the root systems.

Numbers of effective and ineffective *Frankia*, and of myco-nodules among 1454 seedlings from 19 seed-lots were compiled in Table 6. Both mean nodule numbers per nodulated plant and the numbers of nodulated plants are presented. Considerable variation in nodule numbers was observed among ineffective *Frankia* and myco-nodules. While they were found with all seed-lots, ineffective *Frankia* nodules were least numerous on the seed-lots of site 'BB1'. These seed-lots also had the highest number of myco-nodules. Site 'Wie' showed the highest numbers of plants susceptible to ineffective nodulation and also had the highest overall numbers of ineffective *Frankia* nodules.

Table 6 Nodulation data for the seed-lots from each sampling site, with total number of plants, mean number of myco-nodules per plant (between brackets: number of nodulated plants), mean number of effective *Frankia* nodules per plant, mean number of ineffective *Frankia* nodules per nodulated plant.

Seed-lot	Total no. plants	Mean no. of myco nodules / plant	Mean no. of effective nodules / plant	Mean no. of ineffective nodules / nodulated plant
BB1-1	65	2.1 (31*)	1.2 (39*)	1.5 (2*)
BB1-5	78	1.4 (22)	1.5 (60)	7.5 (6)
BB1-6	80	2.4 (37)	1.2 (55)	2.5 (11)
BB1-7	70	2.9 (34)	0.9 (41)	3.5 (16)
Hel-2	84	1.2 (28)	0.5 (31)	7.9 (40)
Hel-3	30	0.3 (1)	0.1 (3)	4.6 (10)
Hel-4	72	0.7 (14)	0.4 (20)	11.3 (11)
Hel-5	46	0.2 (5)	1.0 (21)	8.5 (16)
Zee-1	79	0.8 (17)	1.0 (45)	2.3 (6)
Zee-2	93	1.3 (20)	0.9 (53)	49.2 (43)
Zee-3	48	0.1 (2)	0.8 (18)	12.7 (32)
Zee-4	82	0.5 (18)	1.4 (56)	9.2 (48)
Wie-1	87	0.4 (11)	3.5 (80)	28.8 (52)
Wie-2	84	0.1 (9)	1.0 (48)	9.4 (43)
Wie-3	102	0.4 (17)	2.1 (76)	18.7 (62)
Wie-4	96	0.3 (12)	2.1 (72)	13.9 (60)
BV-1	96	0.1 (5)	1.4 (61)	17.1 (79)
BV-2	84	0.2 (11)	3.0 (78)	6.8 (13)
BV-3	78	0.2 (11)	2.5 (67)	12.6 (25)

*) Number of nodulated plants.

For the calculation of the R-frequencies the mean numbers of ineffective nodules per susceptible plant were used to estimate the chance of occurrence of Poisson zeros. For four seed-lots (BB1-1, BB1-6, BB1-7 and Zee-1) the possibility of zero nodulation due to Poisson distribution could be technically present. In contrast, there were also seed-lots, inoculated with the same soil sample, that showed much higher mean numbers of ineffective *Frankia* nodules per plant. Therefore it was concluded that it was unlikely that zero nodulation due to Poisson distribution caused the low numbers of plants from seed-lots BB1-1, BB1-6, BB1-7 and Zee-1 that were nodulated by ineffective *Frankia*.

The seed-lot R-frequencies of the various sites, and the mean values for each site are presented in Figure 6A. The seed-lots from site 'BB1' had on average the highest R-frequency (87 ± 10) and the seed-lots from site 'Wie' had the lowest average (42 % ± 5). Sites 'Hel', 'Zee', and 'BV' showed moderate R-frequencies (55 - 67 %). The seed-lots from sampling site 'Zee' (55 % ± 26) and especially site 'BV' (57 % ± 35) displayed heterogeneity of R-frequency within the site. The large differences in R-frequencies between sampling sites (Fig. 6A) are in agreement with the

results of the maximum likelihood analyses (Table 7). Seed-lots from sites with an indigenous soil population of ineffective *Frankia* ('BB1', 'Hel' and 'Zee') had mean R-frequencies equal to or slightly higher than the seed-lots from sites without an ineffective *Frankia* soil population ('Wie' and 'BV'). This was also shown by the contrast analyses. Some of the variation in the R-frequency between the sampling sites could be attributed to the absence or presence of an ineffective *Frankia* soil population, but most of the R-frequency variation among these seed-lots could be attributed to site 'BB1' and to site 'Wie'.

Table 7 Results of maximum likelihood analyses and contrast analyses of the percentage of seedlings, resistant to nodulation by ineffective *Frankia* (R-frequency) of the various seed-lots with sampling site, and seed-lot (within sampling site) as the main effects. The contrast analyses compare the impact of the individual sampling sites, and of groups of sampling sites on the R-frequencies. For each analysis the degrees of freedom (DF) are given. Significance levels are indicated as ***: P<0.001; **: P<0.01; *: P<0.05, ns; not significant.

Seed-lot R-frequencies	R-frequency	
	DF	χ^2
Effect:		
Total Data Set:		
Sampling site	4	166.70 ***
Seed-lot (within sampling site)	14	213.21 ***
Contrasts:		
Sampling sites BB1, Hel and Zee vs. Wie and BV	1	68.99 ***
Sampling site BB1 vs. Hel, Zee, Wie, and BV	1	105.25 ***
Sampling site Hel vs. BB1, Zee, Wie, and BV	1	0.67 ns
Sampling site Zee vs. BB1, Hel, Wie, and BV	1	3.51 ns
Sampling site Wie vs. BB1, Hel, Zee, and BV	1	87.04 ***
Sampling site BV vs. BB1, Hel, Zee, and Wie	1	7.00 **

Thus, the statistical analysis indicated that sites 'BB1' (due to its high mean R-frequency) and 'Wie' (due to its low mean R-frequency) differ from the other seed-lot sampling sites. When nodulation data, in the form of shares of effective *Frankia*-, ineffective *Frankia*, and myco-nodules per plant per sampling site were subjected to maximum likelihood analyses, again, site 'BB1' differed considerably from the other sites, particularly with respect to nodulation by ineffective *Frankia* and by *P. nodositatum*. Again, the contrast analysis of the three sites with a detectable ineffective *Frankia* population versus the two sites without ineffective *Frankia* explained much less of the total variation in nodulation found between the sampling sites (data not

shown). In Figure 6B, nodulation values for the sampling sites are presented as arithmetic averages of the shares of ineffective, effective, and myco-nodules per plant per seed-lot. The error bars represent the standard deviations for the mean nodulation values per seed-lot. Figure 6B illustrates the different nodulation behaviour of the seedlings from site 'BB1' compared to the other sampling sites.

Chi-square tests using contingency tables showed that there was a significantly lower number of plants with both ineffective *Frankia* nodules and myco-nodules than was to be expected, based on the distribution of the myco-nodules over the total data set (Table 8). This not only held true for the data set as a whole, but also could be seen when analysing the data for the sampling sites separately; for three of the five sites the difference was significant (sites 'Zee', 'Wie' and 'BV'), and with a fourth site almost significant (site 'BB1'). Calculation of the mean R-frequencies for the sampling sites after omitting plants with myco-nodules showed no significant change from the R-frequencies given in Table 6, which indicates that myco-nodulation does not interfere with ineffective *Frankia* nodulation (data not shown). While in the case of site 'BV', an interaction between effective and ineffective *Frankia* nodules was found, this was not the case for the complete data set. There was no evidence of interaction between effective *Frankia* and myco-nodules.

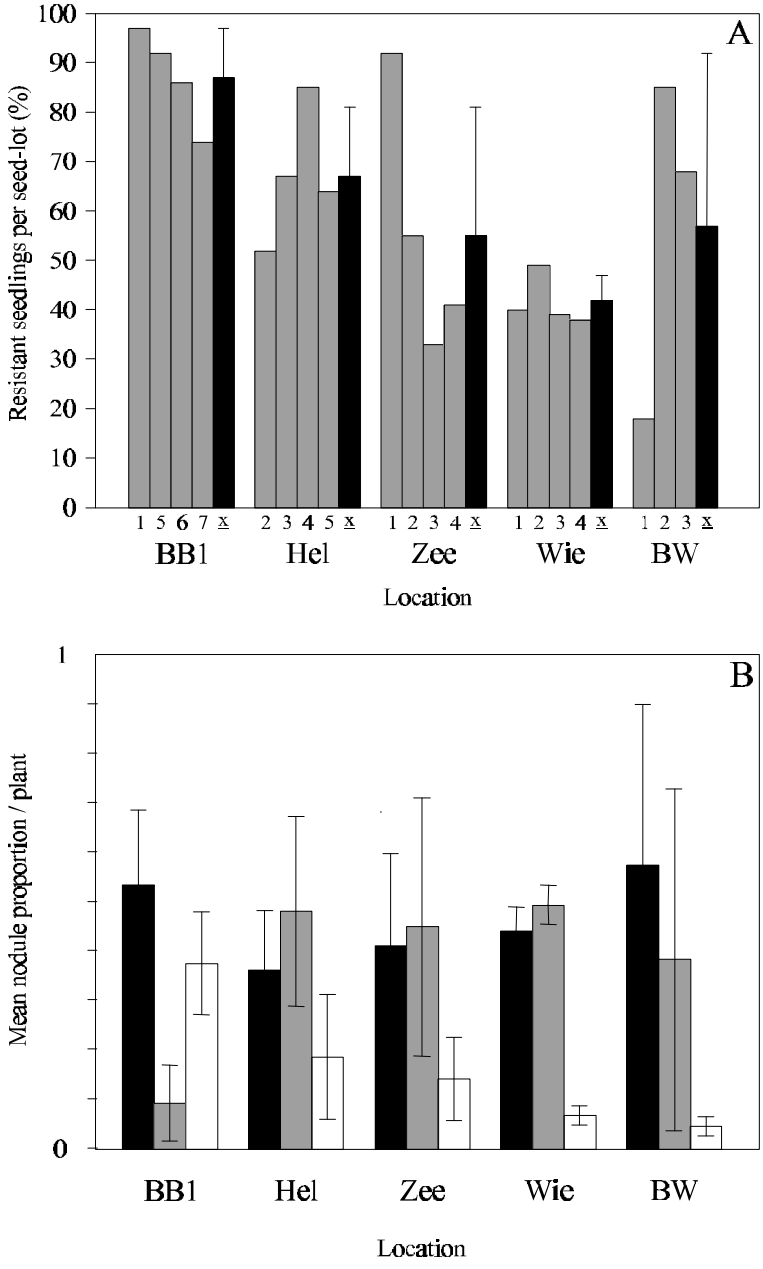


Figure 6 A: Percentage of seedlings resistant to nodulation by ineffective *Frankia* of the individual seed-lots for sampling sites 'Boezem van Brakel 1 (BB1)', 'de Hel' (Hel), 'Zeegsersteeg' (Zee), 'de Wieden' (Wie) and 'Burgvallen' (BV), with the mean R-frequency for each sampling site (x). Numbers under each column correspond to the individual seed-lots displayed in Table 6.

B: Nodulation, shown as the mean proportion of effective *Frankia* nodules (solid bars), ineffective *Frankia* nodules (hatched bars) and *Penicillium nodositatum* nodules (white bars) per seedling (as the share of the total number of nodules per plant), calculated over the individual seed-lots within each sampling site. The error bars represent the standard deviations calculated per seed-lot.

Table 8 Results of Chi-square tests to determine the existence of nodulation interference between effective and ineffective *Frankia* (eff. vs ineff.), effective *Frankia* and *Penicillium nodositatum* (eff. vs myco), and between ineffective *Frankia* and *P. nodositatum* (ineff. vs myco). Tests were carried out with the total data set, and with the data from the separate sampling sites. Instead of absolute nodule numbers per plant, only absence or presence of ineffective and effective *Frankia*, and of myco-nodules was used. Significant interactions (at $P < 0.05$) are shown in **Bold**.

	χ^2	P								
Total Data Set:										
Eff. vs ineff.	0.05	0.82								
Eff. vs myco	0.53	0.47								
Ineff. vs myco	**47.19**	**0.00**								
	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P
Sampling site:	BB1		Hel		Zee		Wie		BV	
Eff. vs ineff.	0.07	0.79	0.39	0.53	1.10	0.29	0.07	0.80	**12.67**	**0.00**
Eff. vs myco	0.76	0.38	0.00	0.98	0.00	0.99	2.70	0.10	0.05	0.82
Ineff. vs myco	1.93	0.16	0.10	0.75	**8.33**	**0.00**	**4.70**	**0.03**	**6.51**	**0.01**

For the majority of the seed-lots, the effective *Frankia* nodules were found to be Poisson distributed, while this was never the case for the ineffective *Frankia* nodules or for the myco-nodules. In the case of ineffective *Frankia* it was found that much of this was caused by a skewed distribution of plants with large numbers of ineffective nodules (i.e. $x > 10$ plant^{-1}), as opposed to the middle range ($0< x <10$ plant^{-1}). For the myco-nodulation no clear pattern could be seen.

Clonal growth of *A. glutinosa*

Direct cloning of stem sections from the mature *A. glutinosa* trees using methods described in the literature (Périnet *et al.* 1988, Tremblay and Lalonde 1984) proved to be unsuccessful, mostly due to uncontrollable microbial infections. While trying several methods (see also chapter 3a), it appeared that the factor most influencing cloning was the origin of the cloning material, as the only successful rooted clones were obtained from one and the same tree using different cloning methods. As cloning and rooting of greenhouse-grown alder seedlings is generally more successful, the two-step cloning method using 'root-clones', as described in the Materials and Methods section, was applied. Unfortunately, this method also had some drawbacks. Major bottlenecks were finding suitable starting material from each prospective tree, and the long time needed for the two-step cloning. Moreover, the mortality rate of about 90% was still quite high. Among the clones from sites 'Zee', 'Hel' and 'BB1' that were obtained, only two were found to

be susceptible to nodulation by ineffective *Frankia* (see Table 9). The high degree of resistance among alder clones from sites 'BB1' and 'Hel' is in agreement with the high seed-lot R-frequencies from these locations.

Table 9 Results of inoculation experiments with rooted clones of *Alnus glutinosa* trees from sampling sites 'Boezem van Brakel 1', 'de Hel' and 'Zeegsersteeg'.

Sampling site:	Rooted cutting from parental tree:	Reaction with ineffective *Frankia*:
Boezem van Brakel 1	BB1-2	resistant
	BB1-3	resistant
	BB1-4	resistant
De Hel	Hel-1	resistant
	Hel-6	resistant
	Hel-7	resistant
	Hel-8	susceptible
	Hel-9	resistant
Zeegsersteeg	Zee-5	resistant
	Zee-5	susceptible

Search for ineffective *Frankia* nodules on field-grown alder roots

At site 'Zeegsersteeg', young alder roots were mainly found just above the water-table, and all trees examined (except Zee-7) were close to the water's edge. Inoculation trials using cuttings had shown tree Zee-7 to be susceptible to nodulation by ineffective *Frankia*, but this tree yielded few surface roots, none of which was nodulated by *Frankia*. As expected, tree Zee-5, identified as being resistant to nodulation by ineffective *Frankia*, was only nodulated by effective *Frankia*. The root-systems of trees Zee-8, Zee-9 and Zee-11 were also free from ineffective nodulation and on the roots from trees Zee-6 and Zee-10 no nodules were found at all.

DISCUSSION

The partial resistance of *Alnus glutinosa* seed-lots against the nodulation by ineffective *Frankia* may not to be entirely unique among actinorhizal interactions (Baker *et al.* 1979, Torrey and Racette 1989), but it is easily the best studied. Although there is no apparent negative effect on nodulated alder seedlings, these ineffective *Frankia* strains, instead of being symbionts, exhibit characteristics of a (mild) pathogen (Van Dijk and Sluimer 1994). This is confirmed by previous findings of Guan *et al.* (1996), who reported the presence of high amounts of phenolic compounds in the infected cells of ineffective *Frankia* nodules at an early stage of nodule development. In contrast, such an accumulation of phenolic compounds, often seen as a plant response to (fungal) infections (Heath 1991), was not found in plant cells of effective *Frankia* nodules. It was reported that co-inoculation of *A. glutinosa* with both effective and ineffective *Frankia* strains stimulated effective *Frankia* nodulation, and subsequent plant growth (Hahn *et al.* 1990b). Similar nodulation enhancing properties have also been described for *Pseudomonas cepacia* (Knowlton and Dawson 1983), however, and evidence exists that growth stimulation is independent of the type of organism used (Knowlton *et al.* 1980). In contrast with this positive interaction, Van Dijk and Sluimer-Stolk (1990) reported a density dependent reduction of ineffective nodulation by effective *Frankia*. In the present paper, no evidence for interaction between nodulation by effective and ineffective *Frankia* was found, but this may have been largely caused by the low nodulation potential for effective *Frankia* of the soil used for inoculation.

The presence of myco-nodules on the roots of *Alnus glutinosa* has been reported earlier (Van Dijk 1984, Capellano *et al.* 1987, Van Dijk and Sluimer-Stolk 1990). Negative interactions with effective *Frankia* were demonstrated in nodulation experiments (Van Dijk, unpublished data). The possibility of interaction with *Frankia* nodulation has also been postulated by Capellano *et al.* (1987) and by Akkermans *et al.* (1989), which is confirmed by the negative interaction of ineffective *Frankia* and *P. nodositatum* with respect to the nodulation of alder seedlings reported in this paper. In this instance, no interaction between myco-nodulation and the nodulation of effective *Frankia* was found, but again, this may have been caused by the low nodulation potential of the soil inoculum for effective *Frankia*. The exact nature of the observed negative interaction between myco-nodulation and ineffective nodule formation is presently unknown. Competition for

infective loci on the plant roots is a possibility since both (ineffective) *Frankia* and *P. nodositatum* (Sequerra *et al.* 1994) enter *Alnus* roots by means of root hair infection. Omitting plants with myco-nodules from the data-set had no significant effect on the calculated R-frequencies, indicating that while there is a negative interaction between ineffective *Frankia* and myco-nodulation, the main results of the experiments of this paper were not affected. As with ineffective nodulation, no interaction between effective *Frankia* and myco-nodulation was found. This was probably due to the low numbers of effective *Frankia* root nodules that were produced by this particular soil sample, ruling out any effective competition.

The relationship between the R-frequency of a seed-lot and the phenotype of the parent tree is not clear. Presently, from alder trees no data on both the seed-lot R-frequency and the phenotype with respect to ineffective *Frankia* nodulation (**R**esistant or **S**usceptible) is available. Preliminary results of cross-pollination experiments using **R** and **S**-type alder trees indicate that several genes are involved in the determination of these plant phenotypes (C. van Dijk, unpublished data). It was hypothesised that the seed-lot R-frequency and the parent tree's resistance to ineffective nodulation are closely linked. Would ineffective *Frankia* be solely dependent on the nodulation of alder trees for growth and maintenance of population size, generally a low R-frequency would be expected for alder sites with a large soil population of ineffective *Frankia*. As the resistance data for the seed-lots of the three sampling sites supporting an ineffective *Frankia* population do not show a low percentage of resistant seedlings, such dependency on intimate symbiotic interactions is unlikely to be true. This conclusion is reinforced by the low number of trees, susceptible to ineffective *Frankia* nodulation, that were found through the inoculation experiments with cloned alder plants. Also, at location Zeegsersteeg, no ineffective *Frankia* nodules were found on the roots of any of the investigated alder trees.

The R-frequencies for the sites with an ineffective *Frankia* soil population seemed as high or higher than the R-frequencies of the sites without ineffective *Frankia*. This could be interpreted as the result of selection for resistance among the alder individuals at these locations, especially in the light of the significantly higher R-frequencies found for the seed-lots of site Boezem van Brakel 1, which is also the source of the soil inoculum used in the experiments described in this paper. Whether this is indeed the result of local adaptation, or not, is uncertain. It would mean that the

extent of resistance within an *A. glutinosa* seed-lot is partly determined by the origin of the ineffective *Frankia* used for the R-frequency assessment. Before further conclusions can be drawn, cross-inoculations of the same seed-lots with soils from the respective sampling sites would have to be carried out.

In conclusion, ineffective *Frankia* do not seem to be absolutely dependent on the development of ineffective nodules on the host plant *A. glutinosa* for the maintenance of their population size. Still, an interaction with *A. glutinosa* based on saprophytic growth or superficial infections may not yet be disregarded. While a negative interaction between the nodulation of ineffective *Frankia* and *P. nodositatum* was found, this did not affect the seed-lot R-frequencies.

Chapter 4

PHYLOGENY OF INEFFECTIVE *FRANKIA* STRAINS FROM *ALNUS GLUTINOSA*

4a: Phylogenetic tree construction

INTRODUCTION

Since the late 1980s, the use of phylogenetics for the description of genetic variation and species interactions has increased greatly. This increase can be chiefly attributed to the development of the Polymerase Chain Reaction (PCR) technique for the amplification of specific DNA fragments. The advent of PCR techniques, personal computers, and the use of automated DNA sequencing machinery has made the ability to amplify, determine, and analyse DNA sequences widely available. This 'technological revolution' has consequently lead to a great increase in the number of available DNA sequences. In recent years, phylogeny as a descriptive and analytic tool has become more and more appreciated (Hillis 1997). Also in the field of microbial ecology (Head *et al.* 1998), and especially in the field of *Frankia* research, phylogeny has become a valued addition to the traditional methodology. The power of phylogenetic data has been demonstrated by the revaluation of the relationship between all symbiotic, nitrogen-fixing nodule-bearing plants (Soltis *et al.* 1995). This extensive research demonstrated that these plant species, both leguminosae and actinorhizal plants formed one monophyletic group. It also appeared to be possible to correlate the various morphological and physiological differences between certain groups of actinorhizal plant/*Frankia* combinations, and their lines of descent (Swensen 1996).
Molecular phylogenetic methods made it possible to study uncultured and/or slow growing organisms, and from this, the research of *Frankia* has also benefited.

While DNA sequences can be obtained fairly easily, the variety in and the number of tree-construction methods may seem staggering, and existing phylogenetic software usually does little to clarify matters. In this section, some of the principles forming the basis of phylogeny (for further reading: Felsenstein 1988, Miyamoto and Cracraft 1991, Nei 1997) are described, as well as some of the most widely used methods offered in contemporary phylogenetic software packages.

Chapter 4

PHYLOGENY

The Data Set

The choice of the target DNA for the phylogenetic analysis is determined by the relative relatedness of the operational taxonomic units (OTU's; populations, species, or strains). When the OTU's are expected to be closely related, DNA sequences with a high mutation rate (variable regions) should be considered, while comparison of distantly related organisms would need more conserved sequences.

The ribosomal RNA genes are widely used for phylogenetic purposes (Woese 1987). The rRNA genes, coding for the small and large rRNA sub-units, are present in all living organisms and contain both conserved and variable regions. This makes these genes useful all-round targets for the construction of phylogenetic trees. As a consequence, much rRNA sequence information is already available in the various DNA data bases (Ludwig 1995), such as the Ribosomal Database Project (RDP; rdp.life.uiuc.edu), the European Bioinformatics Institute (EBI; ftp.ebi.ac.uk) Databases and GenBank (NCBI; ncbi.nlm.nih.gov). The RDP database exclusively holds ribosomal nucleotide sequences, but the others are more general databases, containing nucleotide sequences from a wide range of genes, as well as amino acid sequences from proteins (SWISS-PROT, one of the EBI databases). Protein-coding gene sequences are particularly useful when conducting research on groups of organisms, which perform specific functions within an ecosystem.

Traditionally, phylogeny is determined by scoring for the presence or absence of homologous morphological or physiological characters across taxa. These characters are placed in a data matrix, which form the basis of the phylogenetic comparison. While morphological or physiological characters are still used for inferring phylogeny, for instance in the field of palaeontology (i.e. Sereno *et al.* 1994), DNA nucleotide sequences are also used. When using DNA sequences, each nucleotide position can be considered to be a separate character, with four possible states (one for each nucleotide base), and an arbitrary fifth state (a gap; i.e. the absence of a homologous nucleotide base). Consequently, a correct alignment of homologous nucleotides of the DNA sequences is a crucial requirement for any successful phylogenetic analysis . The best

alignment algorithms use phylogenetic principles to determine sequence homology, as opposed to simply comparing nucleotides (Mindell 1991, Waterman *et al.* 1991).

Changes in the nucleotide sequence can either occur due to nucleotide substitutions, insertions, or deletions. The nucleotide bases can be divided into the spherically large purines (Adenine and Guanine) and the smaller pyrimidines (Cytosine and Thymine). In the DNA helix, a purine is coupled by H-bridges to the corresponding pyrimidine base (A with T, and G with C), resulting in the constant 10 Å radius of the DNA helix. Mutations resulting in base substitutions can be of two kinds. Transitions replace a purine with a purine (G for A or vice versa) or a pyrimidine for a pyrimidine (T for C or vice versa). Transversions replace a pyrimidine with a purine or vice versa. As the latter type of substitution spherically distorts the DNA helix, it is recognised more easily by DNA repair mechanisms and therefore occurs less frequently (Kimura 1980). Differential nucleotide substitution rates can be modelled and taken into account when calculating evolutionary distances.

When rates of substitutions are equal between OTU lineages, it is said a molecular or evolutionary clock is operating. It is known, however, that in some cases these rates may vary among lineages (Britten 1986). For protein-coding genes, the rate of substitution of the third triplet nucleotide position is usually greater than the substitution rates of the first two triplet positions (Shoemaker and Fitch 1989).

If necessary, gaps are to be introduced in places where deletions or insertions have occurred in the course of evolution. The cost of introducing a gap in an alignment is generally rated higher than a base substitution. Gap elongation penalties are intermediate between these two values. This could, for instance, result in one large gap as opposed to two, smaller, adjacent gaps. While introducing gaps may be necessary for creating a correct sequence alignment, the occurrence of insertions and deletions is rather haphazard as compared to nucleotide substitutions. Consequently, gap positions are often omitted from further tree construction calculations.

For many of the phylogenetic methods, an out-group OTU is required. The outgroup should be a taxon which falls outside the phylogenetic clade to be analysed, but one which is not too distant so as to disrupt the sequence alignment. The choice of this out-group can have a significant influence on the quality of the sequence alignment, especially when nucleotide substitution rates may vary between lines of descent.

When constructing a phylogenetic tree, a distinction has to be made between species trees and gene trees (Tateno *et al.* 1982). In a species tree, ancestral species branch into contemporary species, representing the evolutionary pathway of these organisms. A gene tree is constructed using only part of the genome of the species, and may, depending on the gene or gene-fragment used, differ from the actual species tree (Nei 1991).

Tree Making Methods

For the construction of a phylogenetic tree, DNA data can be divided into two categories. In the first group, the sequence information is reduced to evolutionary distance values calculated for each pair of OTU's. These values are put in a mathematical matrix, also called a *Distance Matrix*. The phylogenetic tree is then constructed through comparison of these distance values. Various algorithms can be used for the calculation of the distance matrix and the subsequent creation of the phylogenetic tree. The issues raised in the DNA alignment section (see above) about nucleotide substitution rates and gap penalties also apply here.

In the second category, the sequence information is not condensed into distance values, but the character states of the nucleotide positions are considered separately. Therefore, this type of data is called *Discrete-Character* data.

For tree construction using Distance Matrix data and Discrete-Character data several different methods are available.

Distance Data

While distance data can be obtained using techniques like DNA-hybridisation, they can also be derived from DNA sequences. There are a number of different models that are used to calculate distance matrices of DNA sequences, most of which correct for superimposed mutations (i.e. substitutions of substituted nucleotides).

The oldest and most widely used is the model of *Jukes and Cantor* (1969) which makes no distinction between the different nucleotide substitutions for the distance calculation. The model of *Tajima and Nei* (1984) makes a distinction between nucleotide substitution rates, but does not correct for nucleotide transitions and transversions. The latter corrections are used, however, in

the model described by *Kimura* (1980). All of these methods are only approximations of the actual nucleotide substitution patterns.

The distance matrices are then used to calculate the distance tree. Again, several methods are known, many of which utilise cluster analysis. These methods pair the two least distant OTU's into one node. Every subsequent step consists of further linking two sequences or nodes into one new node, until only two nodes remain. Such models include *WPGMA* (Weighted Pair Group Method Using Arithmetic Averages), *UPGMA* (Unweighted PGMA, Sokal and Michener 1958), *Single Linkage* and *Complete Linkage* methods.

Instead of clustering, methods such as that of *Fitch-Margoliash* (Fitch and Margoliash 1967) utilise a pair-wise approach to constructing phylogenetic trees. The Fitch-Margoliash algorithm, unlike UPGMA, does not assume an evolutionary clock to be present.

The *Minimum Evolution* method (Cavalli-Sforza and Edwards 1967) computes the sum of all branch lengths for all possible tree topologies. The topology with the smallest combined branch length is chosen as the final tree. The *Neighbour-Joining* method (Saitou and Nei 1987) uses a simplified algorithm to calculate the tree topology and branch lengths.

Discrete Character Data

Discrete-character data can be handled in two different ways. The first method is based on the 'Principle of Minimum Evolution', which states that the transition from one species to another occurs in the smallest number of mutations possible. Thus, it tries to construct a tree which minimises the overall number of changes necessary to evolve the sequence being analysed. As this method favours the shortest, or most parsimonious tree, it is referred to as the *Maximum Parsimony* principle (Edwards and Cavalli-Sforza 1963). Of the sequence data, only 'informative sites' - nucleotide positions, that are different from that of the out-group OTU, but shared by a part of the sequences - are used. A number of different Maximum Parsimony variants are used, such as Wagner parsimony, Fitch parsimony, Dollo parsimony, and Camin-Sokal parsimony, and they vary mainly in the constraints set upon the changes of sites from one possible character state to another. Dollo parsimony, for instance, does not allow convergent evolution, which excludes identical mutations that occur in divergent lines of the phylogenetic tree.

Unlike maximum parsimony, a second method uses statistical models to calculate the probability with which one sequence is transformed into the other through mutation over time. Instead of using only 'informative sites', this *Maximum Likelihood* method (Felsenstein 1981) utilises all nucleotide positions of the sequence data.

COMPARISON OF TREE-CONSTRUCTION METHODS; ADVANTAGES AND DISADVANTAGES

Since phylogenetic trees represent approximations of the past (family) relationships between the OTU's, it is not possible to assess with absolute certainty whether a given phylogenetic tree is the correct one. The fact that the actual branching pathway is not known means that it is difficult to compare phylogenetic tree-making methods using data sets from contemporary organisms.

In order to fill this knowledge gap, several artificial data-sets have been produced. One method involved the cultivation of (fast-mutating) viral strains, creating known lines of descent of distinct genotypes (Hillis *et al.* 1994). These viral data sets were examined with the UPGMA, the Neighbour-Joining, and the Maximum Parsimony methods. Another method utilised a computer to create an artificial data set, calculating derived sequences from an initial DNA sequence using a series of simulated mutation cycles with variable rates (Nei 1991). Such a computer-generated artificial data set makes it possible to test the ability of the tree-making methods to recreate the correct phylogenetic tree, with different parameters like the number of nucleotides, variable mutation rates, and mutation speed. Nei (1991) tested a great number of phylogenetic models, including UPGMA, Fitsch/Margoliash, Minimum Evolution, Neighbour-Joining, Maximum Parsimony and Maximum Likelihood methods.

The examination of the viral data sets showed that the UPGMA method was much less likely to reproduce the correct tree than the Maximum Parsimony or the Neighbour-Joining methods, which were comparable in efficiency.

Nei (1991) showed that the efficiency of the various methods is dependent upon the constantness in the rate of evolution, the absolute rate of mutation, and the number of nucleotide substitutions per OTU. The UPGMA method, which assumes a constant rate of evolution for all the OTU's was inefficient when mutation rates were not constant. The Maximum Parsimony was

inefficient when the nucleotide substitution rate was low, and the number of nucleotide differences very large. In this case, the Maximum Likelihood, Minimum Evolution, and Neighbour-Joining methods were preferable over the Maximum Parsimony method.

From a practical point of view, there are some other considerations that can play a role in choosing a certain tree-making method. The Neighbour-Joining method only produces the one tree topology with the shortest branch length, while the Minimum Evolution method calculates all the branch lengths for all possible tree topologies, from which the best one can be chosen. Similarly, the Maximum Parsimony method may produce several equally parsimonious trees.

Another factor in deciding the usefulness of a tree-making method is the amount of time it takes to produce a phylogenetic tree. The Maximum Likelihood algorithm may take a prohibitively large amount of computing time when many sequences are involved in the analysis. Similarly, the Maximum Parsimony method may take a long time to calculate, and this also applies to the Minimum Evolution method. On the other hand, the Neighbour-Joining method, which is an approximation of the Minimum Evolution algorithm, is quite fast.

ASSESSMENT OF THE ACCURACY OF THE TREE TOPOLOGY

While simulation experiments may indicate which tree-calculation algorithm is best under which circumstances, the choice of a superior method does not mean that this will automatically result in the correct phylogenetic tree. A number of methods can be applied to assess whether a given tree has a robust topology. Most of these methods are based on the rearrangement of the data set, either by removal (*Jack-knife*), or replacement (*Bootstrap*; Felsenstein 1985) of data points. Repeating the process of arranging sequences and calculating the tree gives a large number of tree estimates, from which a consensus tree can be constructed. A percentage can be allotted to every branch in this tree, indicating the number of times this particular branching was present in the multiple Bootstrap or Jack-knife tree estimates.

Usually only branches with a 95%, or higher, confidence level are considered significantly robust, at a 5% significance level. The ‘majority rule’ principle (see Weir 1990) considers all groups that appear in a majority (over 50%) of the bootstrap trees.

Chapter 4

EXISTING COMPUTER SOFTWARE FOR TREE CONSTRUCTION

For the construction of phylogenetic trees, a large number of software packages is available. These software packages range from the very simple to the complex. A selection of these software packages is given below:

PHYLIP (Phylogeny Inference Package, Felsenstein 1985)

This package consists of a collection of loosely associated programs, for handling, converting and aligning sequences, as well as a variety of tree-building methods.
As a whole, the package is rather unwieldy and user-unfriendly, but can be used on a broad range of computer platforms.

PAUP (Phylogenetic Analysis Using Parsimony, Swofford 1991)

The PAUP package is a commercial product strictly for Maximum Parsimony tree construction. For this, a large number of parameters may be set, and consequently, this powerful package is one of the most widely used. The Maximum Parsimony algorithms are significantly faster than the ones delivered in the PHYLIP package. Unlike PHYLIP, however, PAUP is, until now only available for the Macintosh computer platform.
The separate program MacClade is usually employed for editing and printing of the phylogenetic trees, that have been calculated by PAUP.

Arb (from Arbor (lat.) = tree; Strunk and Ludwig 1995)

The Arb package is a recent product, developed at the University of München. It combines the versatility of PHYLIP with the sophistication of PAUP. Unlike PHYLIP and PAUP, the Arb package is built around an extensive and up-to-date (ribosomal) DNA-sequence library and a powerful DNA-alignment engine. Furthermore, Arb has built-in graphical functions for the drawing, exporting and printing of phylogenetic trees. Arb can be used for the construction of neighbour-joining trees (although without bootstrapping), and of maximum parsimony trees (a heuristic variant), and also has some of PHYLIP's algorithms embedded into its software. Until recently it only ran on Indy platforms, but a PC version has also become available.

Treecon (Van de Peer *et al.* 1994)

This package is dedicated to distance matrix tree-construction methods, and supports boot-strapping for analysis of the robustness of the tree topology. Treecon is small, fast, easy to use, and can be configured fairly extensively. It has a built-in tree editor, but only rather limited file export-facilities. It only runs on PC platforms, with the latest version compiled for Win32 systems. The latest version runs on Windows ’95, and has improved export facilities (ftp://uiam3.uia.ac.be).

Of these four programs, PAUP and Arb are the most powerful and versatile, but have the great disadvantage of not running on “standard” DOS or Windows computers. Arb is better at editing trees than PAUP, much faster, but not very easy to master. Another disadvantage is that it is only suitable for analysing ribosomal DNA sequences. PHYLIP is available for many operating systems, but is slow and definitely not user-friendly. Treecon, although slightly more limited in its options, uses (among others) the reliable (and fast) Neighbour-Joining tree-construction method. Its main advantages are its small size, speed, ease of use, and the fact that it can be run on virtually every PC. Its greatest disadvantage is the lack of sequence alignment facilities. This is not a great problem if the number of new sequences is relatively small.

After considering the advantages and disadvantages of the various tree-construction methods, the Treecon method was chosen for the task of constructing a phylogenetic tree of ineffective *Frankia* strains, primarily because of its speed and ease of operation.

4b: Phylogenetic characterisation of ineffective *Frankia* in *Alnus glutinosa* (L.) Gaertn. nodules from wetland soil inoculants

D. J. Wolters, C. van Dijk, E. G. Zoetendal and A. D. L. Akkermans

Abstract: Ineffective *Frankia* endophytes were retrieved from various wet soils by using *Alnus glutinosa* clones as trapping plants for the formation of root nodules. No pure cultures could be isolated from these ineffective nodules. Therefore, the phylogenetic position of these endophytes was determined by sequence analysis of cloned PCR products of bacterial 16S rDNA, derived from nodules. The results showed that all nodule endophytes belong to a hitherto undescribed cluster of the *Frankia* phylogenetic tree. The position of these uncultured ineffective *Frankia* nodule endophytes is different from that of the ineffective *Frankia* isolates derived from *A. glutinosa* nodules, even when originating from the same geographic location. This suggests a bias in current isolation techniques.

Molecular Ecology (1997) **6**, 971-981 *)

INTRODUCTION

The genus *Frankia* consists of actinomycetes, which form extensive septate hyphae, have multilocular sporangia and, with some exceptions, are able to fix atmospheric nitrogen inside specialised spherical structures, called vesicles. Most *Frankia* strains are able to form symbiotic structures, the nodules, on the roots of host plants, belonging to a large variety of plant species (Benson and Silvester 1993). *Frankia* which are able to fix nitrogen are designated "effective" strains and, consequently, strains which are unable to fix nitrogen are classified as being "ineffective".

Frankia proved to be difficult to bring into culture. The first isolation of a strain belonging to this genus dates from 1978 (Callaham *et al.* 1978), which is more than a century after the first description of *Frankia* nodules by Woronin in 1866. Although many strains have now been isolated and are kept in culture with relative ease, this does not apply to all *Frankia* strains. Molecular techniques now make it possible to gain insight into the phylogeny of *Frankia*, including these uncultured strains (Akkermans *et al.* 1991). Using the plant-trapping method, Van Dijk and Sluimer-Stolk (1990) demonstrated the occurrence of *Frankia* in the soil of a wet dune valley, which induced obligate-ineffective root nodules on the roots of *Alnus glutinosa* (L.). Plants that were well nodulated by this ineffective strain type, and lacked effective *Frankia* nodules, showed a decreased growth rate. Their leaves turned yellow, whereas plants also nodulated by effective *Frankia* exhibited a normal growth, without any symptoms of N-deficiency. *Frankia* isolate AgI5 was subsequently isolated from the ineffective nodules (A. D. L. Akkermans, unpublished data). Partial DNA sequences of the 16S rDNA of other ineffective *Frankia* isolates were compared with sequences from effective *Frankia* strains and found to be significantly distinct (Hahn *et al.* 1989a). Using these data, a group-specific 16S rDNA probe was developed (EF probe; Hahn *et al.* 1989a) that was used to exclusively detect effective *Frankia* strains. Combined with a general *Frankia* probe (FP; Hahn *et al.* 1990a), the EF probe could then be used to distinguish between 16S rDNA of effective and ineffective *Frankia* strains, including ineffective isolate AgI5 (Mirza *et al.* 1991).

In a previous paper (Wolters *et al.* 1997a, see also chapter 2), we described the results of a survey of ineffective nodule-forming *Frankia* soil populations in wet *A. glutinosa* sites in the

Netherlands. At several of these sites, significant numbers of nodule-forming units of an ineffective *Frankia* strain type were found. The term strain type was used to designate groups of *Frankia* strains, which share one or more characteristics. Like other *A. glutinosa*-derived ineffective *Frankia* strains, they lacked the nitrogen-fixing ability with this host plant species (based on plant appearance), and development of functional nitrogen-fixing structures (vesicles) appeared not to take place inside the ineffective nodules. This was accompanied by a high production of phenolic compounds in the infected nodule cells by the plant, suggesting the induction of a defence mechanism (Guan *et al.* 1996). As isolation of the endophytes from ineffective nodules of *A. glutinosa* proved to be unsuccessful (D. J. Wolters, unpublished data), it was decided to characterise these endophytes by isolating DNA directly from the nodules.

In the present paper, we aim to establish the phylogenetic relationship of these ineffective *Alnus glutinosa* nodule inhabitants by comparison of their 16S rDNA sequences with *Frankia* sequences from the 16S rRNA databases, and with the 16S rDNA of effective *Frankia* nodules from the same locations. This also enables us to establish whether these uncultured nodule endophytes either form a monophyletic group, or are clustered with known effective or ineffective *Frankia* strains.

MATERIALS AND METHODS

Sampling sites

Soil samples were collected at sites Boezem van Brakel 1 (BB1; Dutch State Survey Grid 132.6; 424.0), Zeegsersteeg (Zee; S.S.G. 239.0; 565.9) and Veenendaal - de Hel (Hel: S.S.G. 168.3; 477.1) in the Netherlands (Fig. 7). All sites support an *A. glutinosa* population that grows under permanently water-saturated soil conditions, but they differ considerably in soil organic-matter content, mineral soil components, and pH. A description of these sites and the locations was given in a previous paper (Wolters *et al.* 1997a, see chapter 2a). Site Voorne's Duin, Valley 7 (VD7) is situated in a wet sand dune valley (Van Dijk and Sluimer-Stolk 1990), and is the origin of the nodules from which the ineffective *Frankia* strain AgI5 was isolated (A. D. L. Akkermans, unpublished data). All sites are governed as nature reserves by the National Forest Service in the Netherlands (first three sites) and by the Zuid-Holland Trust for Natural Beauty (latter site).

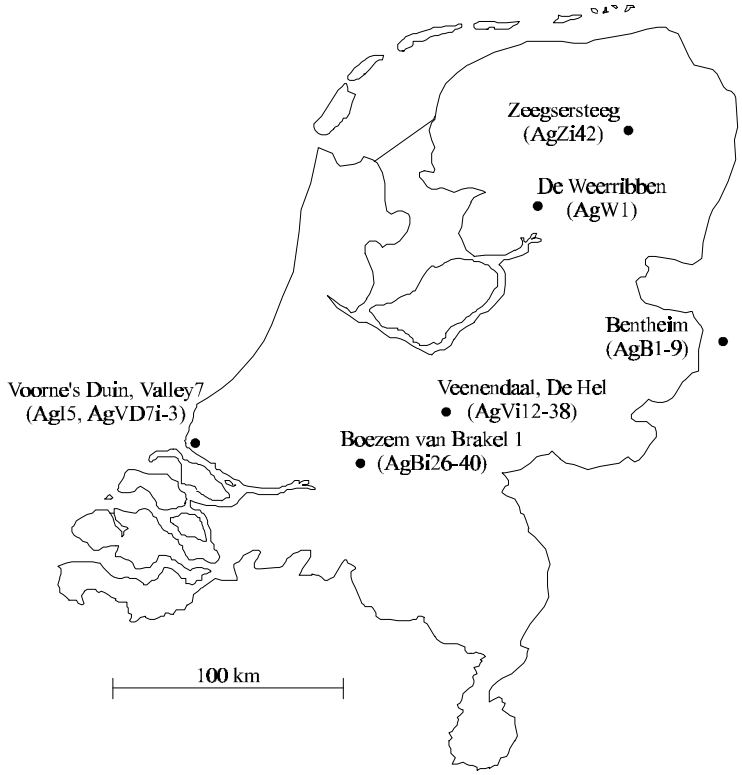


Figure 7 Map of the Netherlands, with geographical origins of ineffective *Alnus*-derived *Frankia* clones and isolates indicated. The names of the areas are given, with the names *Frankia* strains or 16S rDNA clones in parentheses. Also included is the site of origin for ineffective isolate AgB1-9 (Bentheim, Germany).

Frankia nodules

Nodules were obtained using the plant-trapping method. Two to six *A. glutinosa* clones, selected for susceptibility (Hahn *et al.* 1988, Van Dijk and Sluimer 1994) to both effective and ineffective nodulation were obtained using the stem cutting method of Van Dijk and Sluimer (1994, see chapter 3a). Plants were grown for 2 weeks on a modified Hoagland solution with reduced nitrogen content (Quispel 1954a), and inoculated with fresh soil from sites BB1, Zee or Hel at 20 g L^{-1}. After 4-6 weeks, resulting nodules were harvested. Nodule sections were stained with Fabil reagent (Van Dijk and Merkus 1976) and carefully examined for the presence of mature vesicle clusters, which would indicate effective, rather than ineffective *Frankia*. Between examining nodules, cutting equipment was wiped with 70% ethanol. As contrasted to effective

nodules, ineffective nodules were too small to determine nitrogen fixation activity through acetylene reduction assays (dia. 0.5 - 1.0 mm).

Up to 50 ineffective nodules were pooled to reach a fresh weight of 50 - 100 mg in order to obtain sufficient DNA for PCR reactions, and were stored at -70°C. Effective nodule lobes resulting from the same soil samples were stored separately.

Bacterial strains

Escherichia coli strains TG1 and JM109 were used for cloning purposes. Ineffective *Frankia* strains AgI5 (A. D. L. Akkermans, unpublished data) and AgW1 (D. Baker and T. M. Meesters, unpublished data) were grown at 28°C in P+N medium (Meesters *et al.* 1985).

DNA extraction

DNA was isolated from *Frankia* pure strains AgI5 and AgW1 by extraction in phenol using a bead beater, followed by chloroform-isoamyl alcohol (24:1) extraction and ethanol precipitation (Mirza *et al*. 1994b). To extract amplifiable DNA from nodules, 50 - 100 mg of the nodules were first washed in a solution containing 0.2 N NaOH and 1% SDS and rinsed with sterile ultra-pure water. Peeling the ineffective nodules to eliminate the outer layers with possible bacterial contaminants (Benson *et al.* 1996) would have been the preferred technique, but was not feasible due to the small size of the nodules. The nodules were crushed on dry ice (CO_2) in an Eppendorf tube using a micro-pestle. Prior to crushing, large nodule lobes from effective nodules were cut into smaller pieces. DNA was subsequently isolated using cetyltrimethyl ammonium-bromide (CTAB) to remove PCR-inhibiting polyphenolic compounds (Ramírez-Saad *et al.*1996).

PCR amplification

Amplification of 16S rDNA was performed on a Perkin Elmer 480 thermocycler using forward primer PF8*Bam*HI ($^{5'}$CAC GGA TCC AGA GTT TGA T $^{C}/_{T}$ $^{A}/_{C}$ TGG CTC AG) and reverse primer PR1115*Hind*III ($^{5'}$GTG AAG CTT AGG GTT GCG CTC GTT G) (Embley *et al.* 1988). An initial denaturation step of 4 min. at 94°C preceded 35 cycles of 1 min. at 94°C, 2 min. at 48°C, and 3 min. at 72°C, followed by a final post-elongation step of 7 min. at 72°C. Resulting PCR products were purified and concentrated using the QIAquick kit (Qiagen GmbH, Hilden, Germany) prior to cloning.

Nodule DNA extracts were tested for the presence of *nif*H genes with primers $nifH_F$ and $nifH_R$ (Mirza *et al.* 1994a), using the reaction conditions and PCR cycling program as described by Ramírez-Saad *et al.* (1998).

Cloning

PCR products of 16S rDNA were cloned into vector pGEM-T (Promega Corp., WI, USA), according to the Promega manual. The weight ratio of the vector and the PCR product was 2:1. Two microlitres of the reaction mixture were used for transformation in *E. coli* TG1 (Maniatis *et al.* 1982) or in *E. coli* JM109 (Promega 1994). The clones were grown on selective agar plates by standard methods and the plasmid DNA was isolated by using the alkaline lysis method (Maniatis *et al.* 1982).

Hybridization with *Frankia* specific probes

Estimation of the correct size of the clones was achieved through restriction with *Bam*HI and *Hind*III (Gibco, Grand Island, N.Y.). *Frankia*-derived clones were selected through dot blotting. For dot blotting 10 µl of purified plasmid was denatured by incubation for 3 min. at 100 °C, followed by directly cooling on ice. The denatured samples were loaded on a Hybond N+ nylon membrane with a Hybri.Dot manifold (Gibco BRL, Grand Island, N.Y.). The DNA was immobilized with UV light and prehybridized for 15 min. with hybridization buffer, as described by Church and Gilbert (1984). The dot blot was hybridized overnight at 50°C with a [γ^{32}P]-ATP labelled *Frankia*-specific probe (or FP, $^{5'}$ATA AAT CTT TCC ACA CCA CCA G, Hahn *et al.* 1990a) and with effective *Frankia* probe (or EF, $^{5'}$CAG GAC CCT TAC GGA ACC CC). The

blot was washed twice with 2 x (SSC, 0.1 % SDS) and twice with 0.2 x (SSC, 0.1 % SDS) for 15 min each. The washing steps were carried out at temperatures starting at 51 °C and increasing to

Frankia clones from *A. glutinosa* nodules from sites BB1, Hel and Zee were coded AgB, AgH and AgZ, respectively, followed by an "e"or "i" for "effective" or "ineffective", and by a number. The clone from Voorne's Duin, Valley 7 was coded AgVD7i.

Sequencing and positioning of clones

Positive clones were sequenced by automated sequencing (Applied Biosystems 373 DNA Sequencer; Perkin Elmer ABI PRISM^{tm} Dye Terminator Cycle Sequencing Ready Reaction Kit) with standard plasmid primers -21M13f ($^{5'}$TGT AAA ACG ACG GCC AGT), M13rp1 ($^{5'}$CAG GAA ACA GCT ATG ACC) and with the universal 16S rDNA (reverse) primer p900r ($^{5'}$CCG TCA ATT CCT TTG AGT TT). This resulted in partial 16S rDNA sequences of approximately 934 bases (*E. coli.* positions 129-1073). These partial sequences were analyzed for the occurrence of chimerous constructs using the facilities offered by the Ribosome Datebase Project (Maidak *et al.* 1996), and were compared with EMBL data bank and GenBank sequences (see Table 10) using the FASTA algorithm (Lipman and Pearson 1985) for verification. Sequences were aligned using CLUSTAL W (Higgins *et al.* 1992), and adjusted manually. A phylogenetic bootstrap tree (Felsenstein 1985) of 100 replications was constructed using a distance matrix with the neighbour-joining method (Saitou and Nei 1987), while using the method described by Kimura (1980) to correct for multiple base substitutions. These calculations and subsequent graphical representation were carried out by the computer program TREECON (Van de Peer *et al.* 1994).

RESULTS

*Nif*H gene amplification

The PCR amplification of *nif*H genes yielded products of the expected length (approx. 650 bp) with all the DNA samples isolated from effective nodules of locations BB1, Hel and Zee. No *nif*H PCR product was formed with DNA derived from the pooled ineffective *Frankia* nodules from these same areas and from site VD7, while amplification of 16S rDNA from these same samples did occur normally. This indicates that the pooled ineffective nodule DNA is not significantly contaminated with DNA from effective *Frankia* strains.

Cloning

Per transformation reaction 30 - 100 clones were obtained. In the Dot Blot hybridizations only ± 20 % of these clones reacted with the universal *Frankia* probe. All of these FP-positive clones also reacted with the 'effective' *Frankia* probe EF. The rest of the clones only reacted with a universal 16S rDNA probe. One of these FP-negative clones was sequenced to determine whether they were from plant origin, or the result of bacterial origin, other than *Frankia*. From site BB1, three clones from ineffective nodule material and two clones from (different) effective nodules were selected for sequencing. From site Hel, three ineffective nodule clones and one effective nodule clone were taken. Site Zee yielded only one clone from ineffective nodules, and cloning of 16S rDNA from effective *Frankia* nodules of this site failed as the DNA from the effective nodule was unsuitable for PCR reactions.

Sequencing and positioning of clones

All eight clones from ineffective and three clones from effective nodule material, as well as a clone from ineffective *Frankia* strain AgW1 were sequenced (see Table 10). Although the 16S rDNA sequence of AgI5 was not determined, some sequence information (*E. coli* positions 153-204 and 991-1051) of the 16S rRNA gene of this *Frankia* strain was already known (Mirza *et al*. 1992). The non-*Frankia* clone (Acc. X95278) apparently was derived from *A. glutinosa* plastide 16S rDNA, and showed high homology (98%) with an *A. incana* chloroplast sequence from the EMBL databank (Acc. U03555).

The 16S rDNA sequences from *Frankia* nodules all showed high homology with existing *Frankia* sequences (Table 11), and no evidence for the occurrence of chimerous constructs could be found.

Table 10 List of bacterial strains/16S rDNA sequences used in the phylogenetic tree construction, the original host plant, the strain nodulation and N_2 fixation characteristics, the literature reference of the sequence and the sequence accession number (EMBL databank). For non-isolated strains, Ves^+ or Ves^- (presence or absence of mature vesicle clusters) was used, rather than Fix^+ or Fix^-.

Strains designation	Host plant	Characteristics	Reference	Acc. no.
Frankia isolates:				
ACN14a	*Alnus crispa*	Nod^+, Fix^+	Normand and Lalonde 1982	M88466
AcoN24d	*Alnus cordata*	Nod^+, Fix^+	Simonet *et al.* 1984	L40610
AvN17s	*Alnus viridis*	Nod^+, Fix^+	Fernandez *et al.* 1989	L40613
Ag45/Mut15	*Alnus glutinosa*	Nod^+, Fix^+	Hahn *et al.* 1988	X53209
AgKG'84/4	*Alnus glutinosa*	Nod^+, Fix^+	Hahn *et al.* 1989b	L18976
ARgP5	*Alnus rugosa*	Nod^+, Fix^+	Normand and Lalonde 1982	L40612
ArI4	*Alnus rugosa*	Nod^+, Fix^+	Woese, unpublished data	L11307
CeD	*Casuarina equisetifolia*	Nod^+, Fix^+	Normand *et al.* 1992	M55343
AgB1-9	*Alnus glutinosa*	Nod^+, Fix^-	Hahn *et al.* 1988	L40611
AgI5	*Alnus glutinosa*	Nod^+, Fix^-	Mirza *et al.* 1992	
AgW1	*Alnus glutinosa*	Nod^+, Fix^-	this paper	X95393
Cea1.3	*Ceanothus caeruleus*	Nod^-, Fix^-	Ramírez-Saad *et al.* 1998	U72717
Cea5.1	*Ceanothus caeruleus*	Nod^-, Fix^-	Ramírez-Saad *et al.* 1998	U72718
Cn3	*Coriaria nepalensis*	Nod^-, Fix^-	Mirza *et al.* 1992	L18977
Cn7	*Coriaria nepalensis*	Nod^-, Fix^-	Mirza *et al.* 1992	L18982
Dc2	*Datisca cannabina*	Nod^-, Fix^-	Mirza *et al.* 1992	L18978
Ea1-12	*Elaeagnus angustifolia*	Nod^+, Fix^+	Fernandez *et al.* 1989	L40619
Ea1-28	*Elaeagnus angustifolia*	Nod^+, Fix^+	Fernandez *et al.* 1989	L40618
HR27-14	*Hippophaë rhamnoides*	Nod^+, Fix^+	Fernandez *et al.* 1989	L40617

(Table 10 continued)

Unisolated *Frankia*:				
AgBe3122 nod	*Alnus glutinosa*	Nod^+, Ves^+	this paper	Y12840
AgBe3252 nod	*Alnus glutinosa*	Nod^+, Ves^+	this paper	Y12841
AgHe3210 nod	*Alnus glutinosa*	Nod^+, Ves^+	this paper	Y12845
AgBi26 nod	*Alnus glutinosa*	Ves^-	this paper	Y12842
AgBi34 nod	*Alnus glutinosa*	Ves^-	this paper	Y12843
AgBi40 nod	*Alnus glutinosa*	Ves^-	this paper	Y12844
AgHi12 nod	*Alnus glutinosa*	Ves^-	this paper	Y12846
AgHi30 nod	*Alnus glutinosa*	Ves^-	this paper	Y12847
AgHi38 nod	*Alnus glutinosa*	Ves^-	this paper	Y12848
AgVD7i-3 nod	*Alnus glutinosa*	Ves^-	this paper	Y12850
AgZi42 nod	*Alnus glutinosa*	Ves^-	this paper	Y12849
Cea nod	*Ceanothus cearuleus*	Nod^+, Ves^+	Ramírez-Saad *et al.* 1998	U69265
Cn nod	*Coriaria nepalensis*	Nod^+, Ves^-	Mirza *et al.* 1992	L18981
Dc nod	*Datisca cannabina*	Nod^+, Ves^-	Mirza *et al.* 1992	L18979
Dryas nod	*Dryas drummondii*	Nod^+, Ves^+	Normand *et al.* 1996	L40616
Myrica nod	*Myrica nagi*	Nod^+, Ves^+	Normand *et al.* 1996	L40622
Non-*Frankia* isolates:				
Geodermatophilus obscurus	-----	---	Normand *et al.*1996	L40620

Table 11 Distance matrix for taxonomical units. The values on the lower left are distances, expressed as number of substitutions per site (x 100), as described by Kimura (1980). The values on the upper right are the genetical distances as calculated by the DNAdist program of the PHYLIP software package (Felsenstein 1989). The sequences are divided into groups which represent the clusters described in the phylogenetic tree of Fig. 9.

Sequence	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
AcN14a		0.9957	0.9568	0.9860	0.9784	0.9903	0.9827	0.9795	0.9817	0.9870	0.9881	0.9773	0.9763	0.9784	0.9763	0.9763	0.9763	0.9773
ACoN24d	0.43		0.9568	0.9860	0.9784	0.9903	0.9827	0.9795	0.9817	0.9891	0.9871	0.9795	0.9784	0.9806	0.9784	0.9763	0.9784	0.9773
Ag45/Mut15	4.46	4.46		0.9643	0.9568	0.9535	0.9676	0.9643	0.9611	0.9489	0.9654	0.9447	0.9427	0.9481	0.9427	0.9481	0.9492	0.9481
AgBe3122	1.42	1.42	3.66		0.9881	0.9806	0.9903	0.9892	0.9871	0.9750	0.9957	0.9676	0.9666	0.9687	0.9666	0.9763	0.9763	0.9773
AgBe3252	2.19	2.19	4.46	1.20		0.9730	0.9827	0.9924	0.9784	0.9674	0.9860	0.9643	0.9633	0.9655	0.9633	0.9741	0.9741	0.9752
AgBi34	0.98	0.98	4.81	1.97	2.75		0.9795	0.9741	0.9763	0.9815	0.9838	0.9697	0.9687	0.9730	0.9687	0.9709	0.9730	0.9720
AgHe3210	1.75	1.75	3.32	0.98	1.75	2.08		0.9838	0.9871	0.9739	0.9914	0.9665	0.9655	0.9720	0.9655	0.9730	0.9752	0.9741
AgKG'84/4	2.08	2.08	3.66	1.09	0.76	2.64	1.64		0.9795	0.9696	0.9871	0.9654	0.9645	0.9667	0.9644	0.9730	0.9730	0.9741
ARgP5	1.86	1.86	4.00	1.31	2.19	2.41	1.31	2.08		0.9728	0.9881	0.9611	0.9601	0.9676	0.9601	0.9709	0.9730	0.9720
ArI4	1.32	1.09	5.30	2.55	3.34	1.87	2.66	3.11	2.77		0.9783	0.9695	0.9685	0.9728	0.9685	0.9674	0.9696	0.9685
AvN17s	1.20	1.31	3.54	0.43	1.42	1.64	0.87	1.31	1.20	2.21		0.9676	0.9666	0.9698	0.9666	0.9763	0.9773	0.9773
Cea nod	2.31	2.09	5.77	3.33	3.67	3.10	3.44	3.56	4.02	3.12	3.33		0.9989	0.9881	0.9989	0.9665	0.9665	0.9676
Cn nod	2.42	2.20	5.99	3.44	3.78	3.21	3.55	3.65	4.12	3.23	3.44	0.11		0.9871	0.9978	0.9676	0.9676	0.9687
Dc nod	2.20	1.97	5.40	3.21	3.55	2.76	2.87	3.43	3.32	2.77	3.10	1.20	1.30		0.9892	0.9698	0.9720	0.9709
Dryas nod	2.42	2.20	5.99	3.44	3.78	3.21	3.55	3.66	4.12	3.23	3.44	0.11	0.22	1.09		0.9655	0.9655	0.9666
Ea1-12	2.43	2.43	5.40	2.42	2.64	2.99	2.76	2.76	2.98	3.35	2.42	3.45	3.33	3.10	3.56		0.9978	0.9989
Ea1-28	2.43	2.20	5.28	2.42	2.64	2.76	2.53	2.76	2.75	3.12	2.31	3.45	3.33	2.88	3.56	0.22		0.9989
HR27-14	2.31	2.31	5.40	2.31	2.53	2.87	2.65	2.64	2.87	3.23	2.31	3.33	3.21	2.99	3.44	0.11	0.11	
AgB1-9	3.79	3.56	5.77	3.21	2.65	4.13	3.45	2.98	3.67	4.62	2.99	4.84	4.81	4.70	5.06	2.99	2.87	2.87
AgW1	4.35	4.24	5.99	3.78	3.21	4.70	4.01	3.55	4.23	5.20	3.55	5.53	5.50	5.39	5.75	3.67	3.55	3.55
Cea1.3	2.87	2.87	5.04	2.64	2.53	3.43	2.98	2.64	3.32	3.69	2.53	4.02	4.00	4.35	4.24	2.53	2.53	2.42
Cea5.1	2.76	2.76	4.93	2.42	2.42	3.21	2.87	2.53	3.21	3.69	2.42	3.90	3.89	4.24	4.12	2.42	2.42	2.31
Cn3	3.09	2.87	4.46	2.76	2.87	3.43	2.87	2.08	3.20	3.68	2.64	3.67	3.53	3.53	3.78	3.09	2.87	2.98
Cn7	2.99	2.87	4.82	2.31	2.87	3.44	2.53	2.75	3.10	3.57	2.09	4.37	4.35	4.58	4.59	2.31	2.20	2.20
Dc2	4.00	3.89	5.28	3.32	3.43	4.46	3.20	3.53	3.54	4.72	3.21	5.17	5.14	5.03	5.39	2.86	2.75	2.75
AgBi26	1.53	1.53	4.69	2.31	2.42	1.64	2.19	2.53	2.75	2.32	2.20	2.99	3.10	2.64	3.10	2.88	2.65	2.76
AgBi40	1.42	1.42	4.58	2.20	2.30	1.53	2.08	2.41	2.64	2.21	2.08	2.88	2.98	2.53	2.98	2.76	2.53	2.65
AgHi12	1.75	1.75	4.92	2.53	2.64	2.08	2.42	2.75	2.98	2.55	2.42	3.22	3.33	2.87	3.33	3.10	2.88	2.99
AgHi30	1.20	1.20	4.35	1.97	2.08	1.53	1.86	2.19	2.42	2.09	1.86	2.65	2.76	2.31	2.76	2.54	2.31	2.42
AgHi38	1.20	1.20	4.35	1.97	2.08	1.53	1.86	2.19	2.42	1.99	1.86	2.65	2.76	2.31	2.76	2.54	2.31	2.43
AgVD7i3	1.20	1.20	4.35	1.98	1.97	1.53	1.86	2.20	2.42	1.99	1.87	2.66	2.76	2.31	2.76	2.54	2.32	2.43
AgZi42	2.19	2.19	5.38	2.98	3.09	2.53	2.86	3.20	3.43	3.90	2.86	3.67	3.77	3.32	3.77	3.55	3.32	3.43
Ce D	0.98	0.98	4.58	1.97	2.53	1.53	1.86	2.42	1.86	1.87	1.86	2.65	2.76	1.64	2.53	2.54	2.31	2.43
Myrica nod	0.87	0.87	4.46	1.20	1.75	1.42	1.75	1.86	2.31	1.99	1.42	2.09	2.20	1.97	2.20	2.43	2.43	2.31
G. obscurus	7.49	7.49	10.58	8.58	8.83	8.34	8.59	9.06	8.70	8.52	8.21	7.50	7.72	7.60	7.73	8.96	8.72	8.84

(Table 11 continued)

Sequence	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
AcN14a	0.9632	0.9569	0.9720	0.9730	0.9698	0.9708	0.9612	0.9849	0.9860	0.9827	0.9881	0.9881	0.9881	0.9784	0.9903	0.9914	0.9288
ACoN24d	0.9654	0.9579	0.9720	0.9730	0.9720	0.9719	0.9622	0.9849	0.9860	0.9827	0.9881	0.9881	0.9881	0.9784	0.9903	0.9914	0.9288
Ag45/Mut15	0.9447	0.9416	0.9514	0.9524	0.9568	0.9535	0.9492	0.9546	0.9557	0.9524	0.9578	0.9578	0.9578	0.9481	0.9557	0.9568	0.9016
AgBe3122	0.9686	0.9622	0.9741	0.9762	0.9730	0.9773	0.9676	0.9773	0.9784	0.9752	0.9806	0.9806	0.9806	0.9709	0.9806	0.9881	0.9191
AgBe3252	0.9740	0.9676	0.9752	0.9762	0.9720	0.9719	0.9666	0.9763	0.9773	0.9741	0.9795	0.9795	0.9806	0.9698	0.9752	0.9827	0.9169
AgBi34	0.9600	0.9536	0.9666	0.9687	0.9666	0.9665	0.9569	0.9838	0.9849	0.9795	0.9849	0.9849	0.9849	0.9752	0.9849	0.9860	0.9213
AgHe3210	0.9665	0.9601	0.9709	0.9719	0.9720	0.9752	0.9687	0.9784	0.9795	0.9763	0.9817	0.9817	0.9816	0.9720	0.9817	0.9827	0.9191
AgKG'84/4	0.9708	0.9645	0.9741	0.9752	0.9796	0.9731	0.9656	0.9752	0.9763	0.9730	0.9784	0.9784	0.9784	0.9687	0.9763	0.9817	0.9149
ARgP5	0.9643	0.9579	0.9676	0.9687	0.9687	0.9698	0.9655	0.9730	0.9741	0.9709	0.9763	0.9763	0.9762	0.9666	0.9817	0.9773	0.9180
ArI4	0.9553	0.9489	0.9641	0.9641	0.9641	0.9652	0.9543	0.9772	0.9783	0.9750	0.9794	0.9804	0.9804	0.9707	0.9815	0.9804	0.9196
AvN17s	0.9708	0.9644	0.9752	0.9762	0.9741	0.9795	0.9687	0.9784	0.9795	0.9763	0.9817	0.9817	0.9816	0.9720	0.9817	0.9860	0.9223
Cea nod	0.9534	0.9459	0.9611	0.9621	0.9643	0.9578	0.9503	0.9708	0.9719	0.9686	0.9741	0.9741	0.9740	0.9643	0.9741	0.9795	0.9286
Cn nod	0.9536	0.9462	0.9612	0.9622	0.9656	0.9580	0.9505	0.9698	0.9709	0.9676	0.9730	0.9730	0.9730	0.9633	0.9730	0.9784	0.9267
Dc nod	0.9546	0.9473	0.9580	0.9590	0.9656	0.9558	0.9516	0.9741	0.9752	0.9720	0.9773	0.9773	0.9773	0.9676	0.9838	0.9806	0.9278
Dryas nod	0.9513	0.9439	0.9590	0.9600	0.9633	0.9557	0.9482	0.9698	0.9709	0.9676	0.9730	0.9730	0.9730	0.9633	0.9752	0.9784	0.9266
Ea1-12	0.9708	0.9633	0.9752	0.9762	0.9698	0.9773	0.9720	0.9720	0.9730	0.9698	0.9752	0.9752	0.9752	0.9655	0.9752	0.9763	0.9159
Ea1-28	0.9719	0.9644	0.9752	0.9762	0.9720	0.9784	0.9730	0.9741	0.9752	0.9720	0.9773	0.9773	0.9773	0.9676	0.9773	0.9763	0.9180
HR27-14	0.9719	0.9644	0.9763	0.9773	0.9709	0.9784	0.9730	0.9730	0.9741	0.9709	0.9763	0.9763	0.9762	0.9666	0.9763	0.9773	0.9169
AgB1-9		0.9871	0.9795	0.9805	0.9687	0.9751	0.9762	0.9632	0.9643	0.9600	0.9654	0.9675	0.9653	0.9556	0.9610	0.9654	0.9124
AgW1	1.20		0.9752	0.9763	0.9645	0.9687	0.9699	0.9558	0.9569	0.9536	0.9590	0.9612	0.9590	0.9493	0.9547	0.9590	0.9062
Cea1.3	2.09	2.42		0.9989	0.9838	0.9827	0.9752	0.9666	0.9676	0.9644	0.9698	0.9720	0.9698	0.9601	0.9655	0.9687	0.9159
Cea5.1	1.98	2.31	0.11		0.9849	0.9838	0.9763	0.9676	0.9687	0.9654	0.9708	0.9730	0.9708	0.9611	0.9665	0.9698	0.9169
Cn3	3.21	3.54	1.64	1.53		0.9741	0.9667	0.9676	0.9687	0.9655	0.9709	0.9709	0.9708	0.9612	0.9666	0.9676	0.9149
Cn7	2.54	3.10	1.75	1.64	2.64		0.9838	0.9687	0.9698	0.9665	0.9719	0.9741	0.9719	0.9622	0.9654	0.9698	0.9148
Dc2	2.42	2.97	2.52	2.41	3.41	1.63		0.9622	0.9633	0.9601	0.9655	0.9676	0.9654	0.9558	0.9612	0.9622	0.9106
AgBi26	3.78	4.47	3.43	3.32	3.32	3.21	3.88		0.9946	0.9892	0.9946	0.9946	0.9946	0.9860	0.9817	0.9871	0.9202
AgBi40	3.67	4.35	3.32	3.21	3.20	3.10	3.77	0.54		0.9903	0.9957	0.9957	0.9957	0.9860	0.9827	0.9881	0.9213
AgHi12	4.13	4.70	3.66	3.55	3.55	3.44	4.11	1.09	0.98		0.9924	0.9924	0.9924	0.9827	0.9795	0.9849	0.9180
AgHi30	3.55	4.12	3.09	2.98	2.98	2.87	3.54	0.54	0.43	0.76		0.9978	0.9978	0.9903	0.9849	0.9903	0.9234
AgHi38	3.33	3.89	2.87	2.76	2.98	2.65	3.31	0.54	0.43	0.76	0.22		0.9978	0.9881	0.9849	0.9903	0.9256
AgVD7i3	3.56	4.13	3.10	2.99	2.98	2.87	3.55	0.54	0.43	0.76	0.22	0.22		0.9881	0.9849	0.9903	0.9233
AgZi42	4.58	5.15	4.11	4.00	4.00	3.89	4.57	1.42	1.42	1.75	0.98	1.20	1.20		0.9752	0.9806	0.9137
Ce D	4.02	2.53	3.55	3.44	3.43	3.55	4.00	1.86	1.75	2.09	1.53	1.53	1.53	2.53		0.9881	0.9266
Myrica nod	3.56	4.12	3.10	2.09	3.32	3.10	3.89	1.31	1.20	1.53	0.98	0.98	0.98	1.97	1.20		0.9245
G. obscurus	9.33	7.86	8.82	7.50	9.05	9.06	9.54	8.46	8.34	8.71	8.10	7.86	8.11	9.19	7.74	7.98	

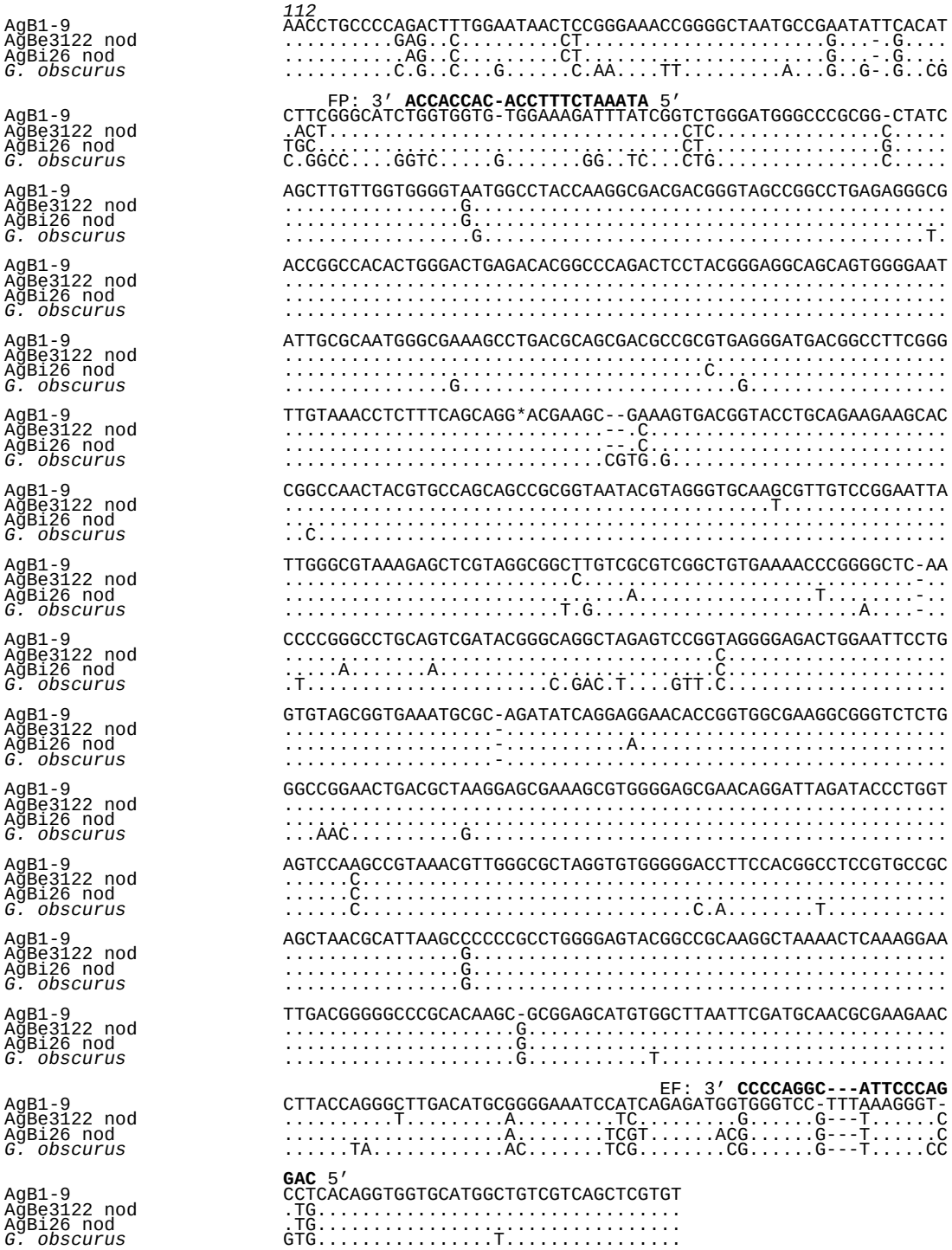


Figure 8 Alignment of some partial 16S rDNA sequences of the *Frankia* strains from Table 10. Represented are AgB1-9 (ineffective *Frankia* isolate), AgBe3122 nod (effective alder nodule), AgBi26 nod (ineffective alder nodule), and *Geodermatophilus obscurus* (outgroup species). The names of nodule-derived sequences are indicated with 'nod'. **Bold** are the universal *Frankia* probe FP, and the 'effective' *Frankia* probe EF, which does not bind to the ineffective *Frankia* isolates. The numbering of the sequence is based on the 16S rRNA of *E. coli*. Identical bases are represented as dots (.), and gaps are indicated with a dash (-).

Although one sequence (AgBi34) grouped with *Alnus* subgroup 1a, the majority of the ineffective nodule sequences that were obtained in this study do not seem to fall into any of the clusters described by Normand *et al.* (1996). As *Alnus* subgroups 1a and 1b in Fig. 9 do not form a monophyletic group, it is proposed to introduce cluster 5 of the *Alnus*-infective cluster to incorporate the ineffective nodule sequences. The effective nodule sequences were incorporated into subgroup 1b (AgBe3122, AgBe3252 and AgHe3210). Previous sequence-data on the rRNA genes of ineffective strain AgI5 (Mirza *et al.* 1992, Hönerlage *et al.* 1994) suggest that this isolate should group with cluster 4 (the ineffective cluster described by Normand *et al.* 1996), as do ineffective isolates AgW1 and AgB1-9, but the ineffective nodule sequence AgVD7i-3 from the same sampling site was more homologous to cluster 5 introduced above (Table 11).

DISCUSSION

The tree shown in Fig. 9 can be divided into the same clusters as described by Normand *et al.* (1996), though with a different branch topology. As Normand *et al.* (1996) argue, branch topology can change considerably between trees when not all parameters (i.e. length of DNA sequence) are exactly the same. A phylogenetic tree constructed using 23S rDNA sequences (Hönerlage *et al.* 1994) shows a similar clustering, with ineffective *Frankia* isolates in a separate cluster from the main body of *Alnus*-infective *Frankia*, which are divided into several groups.

The ineffective, nodule-derived *Frankia* strain type that was found in previous experiments (Wolters *et al.* 1997a) appears to be phylogenetically homogeneous, but different from ineffective alder isolates AgI5, AgW1 and AgB1-9. In fact, AgVD7i-3, a nodule-based sequence from location VD7, clusters with the other ineffective nodule sequences, while it originated from the same location as ineffective isolate AgI5. The results confirm previous observations that nodule derived sequences may differ from those of *Frankia* isolates from the same nodule.

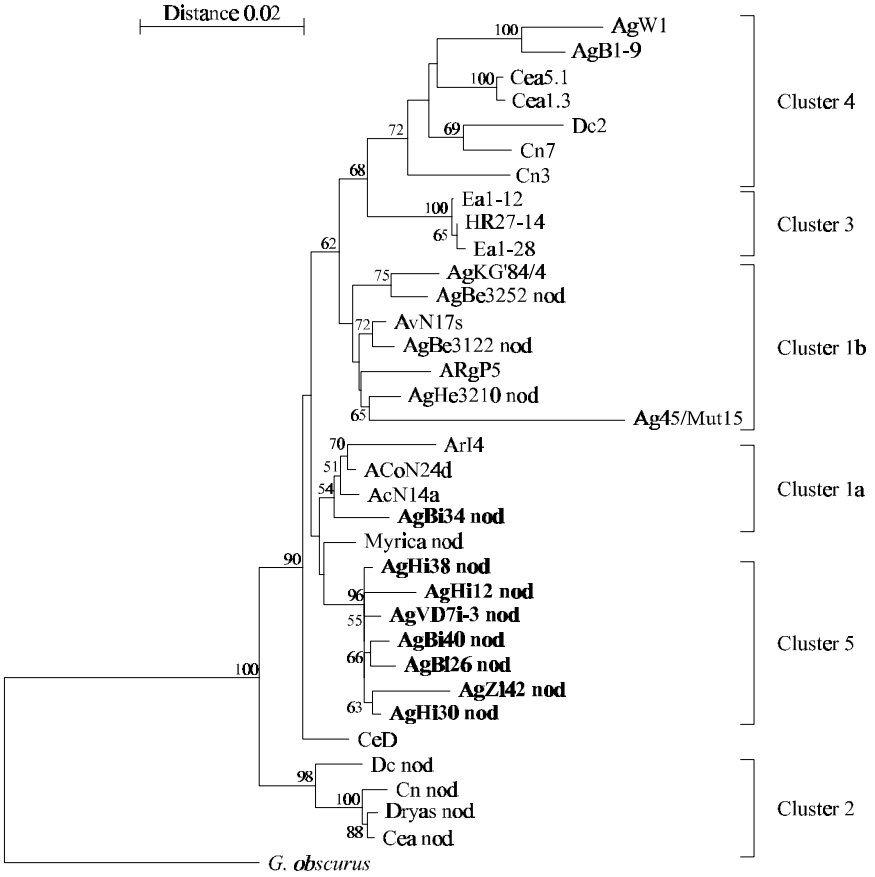


Figure 9 Phylogenetic distance matrix tree, created by the neighbour-joining method (Saitou and Nei 1987). The distance matrix was calculated using the Kimura algorithm (Kimura 1980), and the tree was rooted using *Geodermatophilus obscurus obs.* as outgroup species. The numbers in the tree are bootstrap values, with only numbers greater than 50% shown. For the calculations, a total of 934 sites (including gaps) were used per sequence. The bar represents 0.02 base substitutions per site (s/s). The non-isolated, nodule-derived *Frankia* strains are indicated with **nod**. *Frankia* sequences from ineffective *Alnus* nodules are shown in **Bold**. In addition, sequence clusters are indicated as used by Normand *et al*. (1996).

The results of Mirza *et al.* (1992, 1994a, b) show marked differences between *Frankia* isolates from *Datisca cannabina* (Dc2) and *Coriaria nepalensis* (Cn3, Cn7) nodules, and the sequences derived directly from these nodules (Dc nod, Cn nod). Similar results were found by Ramírez-Saad *et al.* (1998) with respect to *Frankia* strains associated with *Ceanothus caeruleus* (Cea1.3 and Cea5.1; ineffective isolates, Cea nod; nodule sequence), and by Nazaret *et al.* (1989) with respect to *Casuarina* isolates. All of these isolates, for so far they have been sequenced, fall within cluster 4 (Fig.9, Table 11). These results suggest that a bias exists in the current isolation methods, which preferentially isolate strains that are apparently not the dominant ones in the nodule. Nazaret *et al.* (1989) made the same suggestion for *Frankia* isolates from *Casuarina* root nodules. This means, that isolated *Frankia* strains should be examined critically, and when possible, genetically compared to the nodule endophyte. This isolation-bias seems to be less of a problem for the effective sp(-) *Frankia* associated with *Alnus*. These isolates do not form a separate cluster, but group together with nodule-derived sequences (Fig. 9).

While the ineffective nodule endophytes that were the focus of this study did not appear to be closely related to effective *Frankia*, even from the same area, they seem to be more homologous with a *Frankia* sequence derived from (effective) *Myrica nagi* root nodules (Table 11). Whether this means that the ineffective nodule sequences are related to *Myrica* sequences in general will have to be the subject of further study. The fact that no *nif*H genes could be detected in any of the DNA samples from the ineffective nodules supports the hypothesis that these *Frankia* strains are indeed ineffective, although such 'negative' evidence can not be regarded as definite proof.

Ineffective nodule clone AgBi34 nod is different from the other ineffective nodule derived clones, as it groups with strains ACoN24d and AcN14a, within *Alnus* cluster 1a. It may be either derived from a young effective nodule, that was characterised incorrectly, or from a *Frankia* strain on the nodule surface.

The sequences from effective *Frankia* nodules that were determined in this study all group with *Alnus* cluster 1b, and seem to be as diverse as the ineffective nodule sequences (see Table 11). Although due to the pooling of ineffective nodules for template preparation several ineffective *Frankia* strains may have been overlooked, diversity between ineffective nodule sequences from different sampling sites is fairly low, suggesting that these strains do form a tightly grouped cluster.

From the data presented above, it appears that there are at least two different groups of ineffective *Frankia* associated with *Alnus glutinosa*, the first one being represented by isolates such as AgW1, AgB1-9, and AgI5, with the second one represented only by nodule-derived 16S rDNA sequences. The fact that the PCR amplification of 16S rDNA of ineffective nodules from soil from Voorne's Duin, Valley 7 amplified clone VD7i-3 and not an AgI5-type sequence shows that the latter ineffective strain type (the 'isolates'-group) is not the sole inhabitant of ineffective nodules from this location. While the actual numerical importance of either the 'isolates' or the 'nodules'-group is impossible to establish from one nodule sequence, circumstantial evidence suggests that the 'isolates'-group consists mainly of co-symbionts or co-inhabitants of *Frankia* nodules. Nick *et al.* (1992) suggested this for ineffective *Frankia* isolates from *Coriaria* (Cn3, Cn7) and *Purshia* (PtI1; not in Fig. 9, but groups within cluster 4), and this was again postulated by Ramírez-Saad *et al.* (1998) for *Frankia* isolates derived from *Ceanothus*. It must be noted that the situation for ineffective isolates from *A. glutinosa* may be somewhat more complex, as the isolates AgW1, AgI5, and AgB1-9 are able to produce nodules on the roots of *A. glutinosa* seedlings, albeit only when applied at high inoculation densities (D. J. Wolters, unpublished data). The other representatives of this group of *Frankia* all have been reported to be unable to re-nodulate their original hosts.

In conclusion, it can be stated that the hitherto non-isolated ineffective *Frankia* strains form a new monophyletic group within the *Frankia* phylogenetic tree, and can be found in substantial numbers (based on nodulation data) under a wide range of physico-chemical soil conditions in the rhizosphere of wet alder stands.

4c: Comparison of the phylogenetic position of ineffective *Frankia*, as produced by different tree-making methods

INTRODUCTION

One of the main problems encountered in molecular phylogeny is how to test the robustness of a given phylogenetic tree. Choosing a good tree-making method does not necessarily mean it will produce the correct phylogenetic tree. Having a phylogenetic program like PAUP calculate many iterations will give some certainty that one of the most parsimonious trees that are produced is close to the actual tree, and boot-strapping may give some indication of the robustness of the topology of a given tree. There is, however, another avenue of approach, while not as statistically sound as the boot-strapping method, which may lead to a greater sense of understanding of the correctness of a certain tree. This method is based on using several different tree-making algorithms to create phylogenetic trees of the same data set. If a given tree topology is correct, it should be reproduced by most of the tree-making algorithms.

In order to check the *Frankia* 16S rDNA tree topology produced by the Neighbour-Joining method of Treecon (which was used in chapter 4b), a number of other algorithms, described in chapter 4a, were used on a similar data set.

MATERIALS AND METHODS

The Data Set

For the data set, a total of 35 *Frankia* 16S rDNA sequences was used, as well as the 16S rDNA sequence of outgroup species *Geodermatophylus obscurus*. Of the *Frankia* sequences, 25 were taken from the sequence data bank included with the phylogenetic computer program Arb. These sequences, and their accession numbers can be found in Table 10 of the previous section of this chapter, except for strain PtI1 (*Purshia tridentata*; acc. L41048; Normand *et al.* 1996). The other ten sequences were obtained from effective and ineffective *Frankia* nodules induced on roots of *A. glutinosa* seedlings (Wolters *et al.* 1997b, see chapter 4b). The sequences were aligned with the alignment program incorporated into Arb, using the Clustal W algorithm (Thompson *et al.* 1994). Of the 16S rDNA sequence, a total of 934 base positions (equalling the length of the shortest sequence) were compared.

Tree-construction

Of the tree-making methods offered by Arb, the distance matrix methods of Fitch-Margoliash, the De Soete Treefit, and Neighbour-Joining were used. The maximum parsimony program from PHYLIP was also employed. In combination with the Neighbour-Joining method, a number of different methods correcting for superimposed mutations were used, including the methods of Felsenstein, Jukes and Cantor, and the Kimura 2-parameter algorithm.

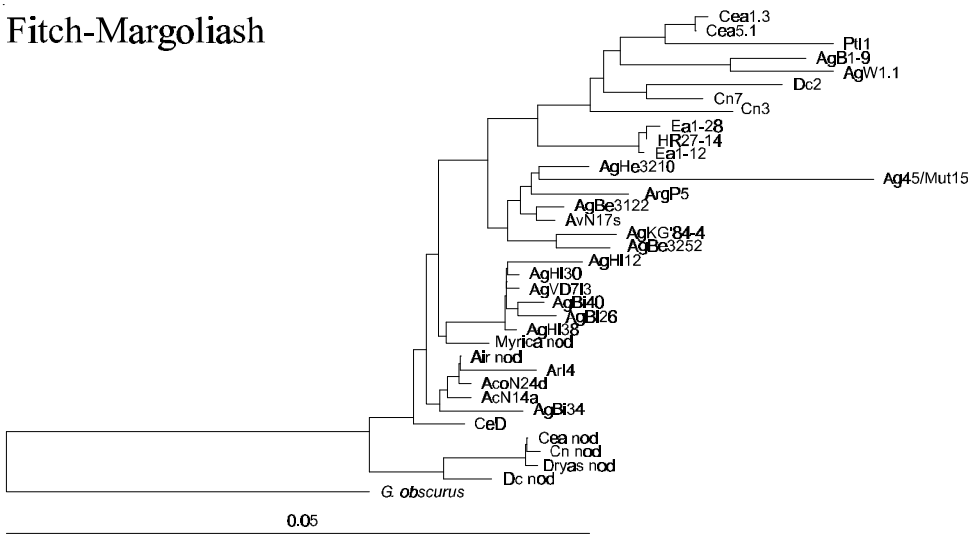
RESULTS AND DISCUSSION

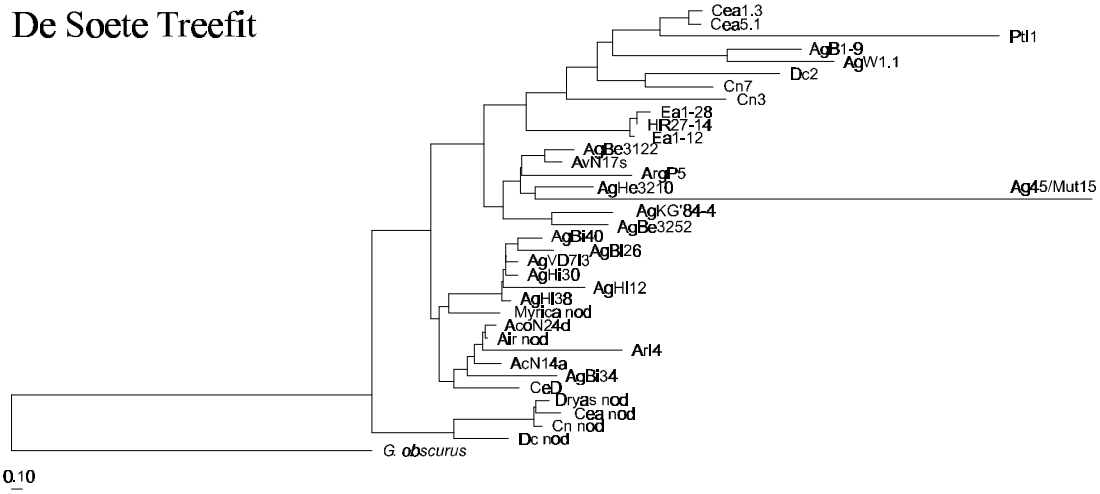
Four different phylogenetic trees, constructed using different algorithms, are displayed in Figure 10. For the Neighbour-Joining trees, it did not appear to matter which algorithm was used for the correction for superimposed mutations: Essentially the same tree was created with every one of these correction methods, although minor differences could be found in the arrangements of sequences within monophyletic groups. Composition and branching of the major clusters of sequences were identical.

The major groups of sequences to be seen in these trees were from a group of non nitrogen-fixing isolates (strains Cea1.3, Cea5.1, PtI1, AgW1.1, AgB1-9, Cn3, Cn7, and Dc2), a group of *Elaeagnus*-infective strains (HR27-14, Ea1-12 and Ea1-28), a group of *Alnus*-infective nitrogen-fixing strains (Alnus I; AvN17S, ArgP6, Ag45/Mut15, AgKG'84-4, AgBe3122, AgBe3252 and AgHe3210), a group of non-isolated *Alnus*-infective non nitrogen-fixing strains (AgBi26, AgBi40, AgHi12, AgHi30, AgHi38 and AgVD7i3), a second group of *Alnus*-infective nitrogen-fixing strains (AcoN24d, ArI4, Air nod, AcN14a and AgBi34), and a group of non-isolated strains from various origins (Cea nod, *Dryas* nod, Cn nod and Dc nod). Finally, two strains (CeD and *Myrica* nod) did not fall into any of the above mentioned clusters.

Between tree-construction methods, the clustering of these sequences remained the same (Fig. 10). Only with the De Soete Treefit, the position of strain CeD, relative to the other sequences was changed. The PHYLIP Parsimony tree did not yield a monophyletic cluster for

the first group of *Alnus*-infective nitrogen-fixing strains, but it did place them close together within the tree. Again, within each cluster, the relative positions of the sequences varied, especially within the cluster of non nitrogen-fixing *Frankia* isolates. In all of the trees, the genetical distances between the sequences in this cluster were large.





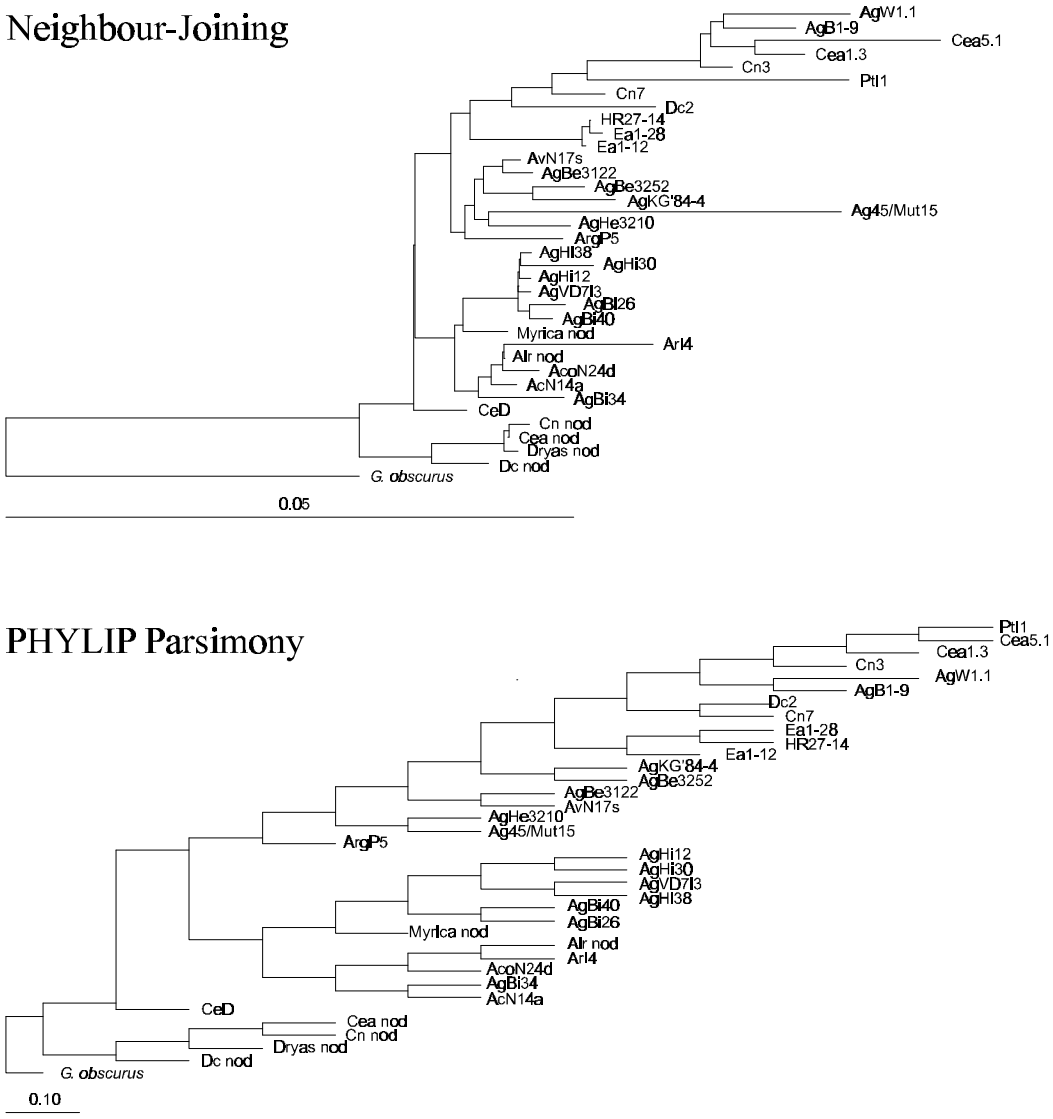


Figure 10 Phylogenetic trees of 35 *Frankia* 16S rDNA sequences, with *Geodermatophylus obscurus* as outgroup species. The phylogenetic trees were constructed with the Arb program, using the algorithms of Fitsch-Margoliash, De Soete Treefit, Neighbour-Joining, and (PHYLIP) Parsimony.

In conclusion, the trees constructed with the various tree-making algorithms were sufficiently similar to demonstrate the robustness of the phylogenetic tree that was shown in the previous section of this chapter (4b, Fig. 9). It also demonstrates, however, that care

should be taken when drawing conclusions about the placement of single sequences within a section of a phylogenetic tree.

Chapter 5

GENERAL DISCUSSION AND PROSPECTS

DISCUSSION

Following the data that were presented in the previous chapters, it is now possible to analyse the three questions addressed in the Introduction (chapter 1):

1) *Do ineffective* Frankia *form a common part of the* Frankia *soil population at wet alder sites, or do they only occur in a limited geographical area?*

In chapter 2 (Wolters *et al.* 1997a) it has been shown that ineffective *Frankia* form an important fraction of the *Frankia* population of wet soils under Black Alder vegetation. Ineffective *Frankia* nodules were formed with soil from six locations out of a total of ten, while effective nodules were found at all of these sites. At three locations the majority of the nodules formed were of the ineffective type. Various physical parameters, as well as the concentrations of several chemical components, were determined, but no correlation was found between any one of these parameters and the distribution of these *Frankia* strains.

Ineffective *Frankia* were initially discovered in an old, wet dune valley with an *A. glutinosa* macro-vegetation, in the south-western part of the Netherlands, near Oostvoorne (Van Dijk and Sluimer-Stolk 1984). A further detailed survey of this location showed that the presence of the ineffective strain type was positively correlated to the soil water-table (Van Dijk and Sluimer-Stolk 1990). In the lower, permanently inundated part of the dune valley, the ineffective *Frankia* were found in high numbers, while in the dryer areas, their numbers were substantially reduced. In contrast, the numbers of alder-nodulating effective *Frankia* were not significantly affected by the water-level of the sampled areas. The second part of chapter 2 presents some additional indirect evidence that the presence of ineffective *Frankia* in the soil is linked to the level of the water-table. Since the experiments, conducted by Van Dijk and Sluimer-Stolk (1990), the sampling location at Oostvoorne has experienced a succession of dry winters. Because of this, the soil water-table has dropped significantly, and only a minor part of the location is still water-saturated. Our subsequent nodulation experiments showed that the once-impressive ineffective *Frankia* soil-population has dropped to zero in the now-dry areas, while at the wet site, ineffective nodulation is still high (chapter 2b). The data reported in these studies strongly support the hypothesis that ineffective *Frankia* are widely distributed in wet alder sites.

2) *Is the resistance, shown by the host plant* A. glutinosa *to nodulation by ineffective* Frankia *influencing the ineffective* Frankia *population, and are ineffective* Frankia *strains solely*

dependent on the formation of ineffective nodules on A. glutinosa *for the maintenance of their soil population?*

Ineffective *Frankia* strains do not only differ from effective strains in their inability to form nitrogen-fixing nodules on *Alnus glutinosa*, but they also demonstrate incompatibility with part of the half-siblings of *A. glutinosa* seed-lots. The incompatibility results in a heterogeneous nodulation of these seed-lots. Some seedlings are fully nodulated by the ineffective *Frankia* strain type, while others remain free from ineffective nodules. Resistance of some alder clones to ineffective *Frankia* nodulation was first recognised by Hahn *et al.* (1989b), and Van Dijk and Sluimer (1994) reported *A. glutinosa* seed-lots with 24 to 85% resistant seedlings. In order to study some aspects of the incompatibility of natural alder population with ineffective *Frankia* nodulation, it was attempted to clone individual trees from several field locations. In addition, alder clones, sensitive to ineffective *Frankia* nodulation were needed for cultivation of ineffective nodules for phylogenetic studies on this particular *Frankia* endophyte. In chapter 3a, a number of different cloning methods are described that were examined for their usefulness with respect to the cloning of *A. glutinosa*. It was found that especially for cloning of naturally occurring alder trees, the existing methods were not efficient, mainly due to fungal infections emerging from the alder tissue. Finally, a sufficiently satisfactory method was found that was based on the transplantation of part of the alder trees into the greenhouse, followed by a secondary cloning step.

Although it is possible that the soil-population size of ineffective *Frankia* is influenced by waterlogged soil-conditions, the resistance or susceptibility of *A. glutinosa* trees might also be influencing the presence of ineffective *Frankia* in the soil. The relationship between the occurrence of ineffective *Frankia* in wet alder stands and the degree of resistance to ineffective *Frankia* nodulation of seed-lots and clones of alder trees of these particular locations was studied by means of soil-inoculation experiments (chapter 3b). It was assumed that the percentage of resistant seedlings within a half-sib seed-lot was representative for the compatibility of the mother tree with ineffective *Frankia* nodulation. If the effect of host plant compatibility would be negligible, alder trees, or their seed-lots at locations with an ineffective *Frankia* soil-population would not need to be compatible with ineffective *Frankia* nodulation.

The average percentage of resistant plants (R-frequency) of the seed-lots from locations with an ineffective *Frankia* soil-population (as was established in chapter 2) was found to be equal to or higher than the R-frequencies from locations without ineffective *Frankia*. The mean seed-lot R-frequency was highest for the same location from which the soil-inoculum for these nodulation tests was taken. These results strongly suggest that ineffective *Frankia* are not strictly dependent on susceptible *A. glutinosa* for population size maintenance, and may even have caused (local) adaptation of the alder populations. It is likely that these microorganisms largely grow as saprophytes in the soil, although their growth requirements still remain unknown, hence our inability to isolate them.

3) *What is the phylogenetic relationship of ineffective* Frankia *strains from wet* A. glutinosa *soils with Fix*$^-$ Frankia *isolates, and with local effective* Frankia*?*

Over recent years, molecular phylogeny has more and more been used in microbial ecology, and has become indispensable for the study of cryptic and uncultured organisms in complex ecosystems. Chapter 4 offers a synthesis of a number of the methods that are currently in use, and provides some guidelines for the use thereof.

Molecular phylogenetic techniques have been applied to *Frankia* before, and a respectable number of 16S rRNA gene sequences of *Frankia* strains are known. Hahn *et al.* (1989a) showed that ineffective *Frankia* strains, isolated from a number of different sources, shared a common 'specific' nucleotide sequence of the ribosomal 16S RNA that was not found in the known effective *Frankia* isolates. Both ineffective *Frankia* isolates derived from *A. glutinosa* and the uncultured ineffective *Frankia* strains that were encountered in various wet alder soils have in common the lack of nitrogen fixation and the phenomenon of partial host plant incompatibility. Chapter 4 examines whether this physiological similarity was matched by a close phylogenetic relationship. Since isolation experiments using a number of different techniques failed to yield *Frankia* isolates from ineffective nodules, a different approach was used. Instead of using *Frankia* isolates, DNA was isolated directly from ineffective *Frankia* nodules that were induced onto susceptible *A. glutinosa* clones, using soil from three of the locations mentioned in chapter 2. The phylogenetic position of these endophytes was determined by sequence analysis of cloned PCR products of bacterial 16S rDNA. The results showed that all ineffective nodule endophytes belong to a hitherto unknown cluster of the

Frankia phylogenetic tree. The position of these uncultured ineffective *Frankia* nodule endophytes is different from that of the ineffective *Frankia* isolates derived from *A. glutinosa* nodules. This even holds true for an ineffective *Frankia* isolate and ineffective *Frankia* nodules originating from the same geographic location. This suggests that there exists a bias in current isolation procedures. Using total DNA extracts of a number of these ineffective *Frankia* nodules, no trace of the *nif*H gene cluster could be found, indicating that these strains are truly unable to fix nitrogen, instead of being merely incompatible with the host plant *A. glutinosa*.

PROSPECTS

Ineffective *Frankia* can form an important part of the total *Frankia* soil population in wet *Alnus glutinosa* stands. There is evidence that soil waterlogging is essential for the maintenance of a nodulating population of these ineffective *Frankia* strains.

It has been established that host plant resistance does not influence the presence or absence of ineffective *Frankia*, making it unlikely that these strains rely on nodulation of *A. glutinosa* for survival in the soil. Contrary to our initial hypothesis, these soil-inhabiting, uncultured ineffective *Frankia* strains are phylogenetically distant from ineffective *Frankia* strains that have been obtained in culture. The fact that nodulation resistance of *A. glutinosa* occurs with members of both groups of ineffective *Frankia*, indicates that this must be a common response of the host plant towards undesirable inhabitants.

Many questions regarding ineffective *Frankia* can still be raised and, consequently, a great deal of research can still be carried out with respect to ineffective *Frankia* soil populations.

What is the reason that ineffective Frankia *strains are dependent on soil water-logging?* Since no ineffective pure cultures from this particular group of ineffective strains are available to date, this question is difficult to answer at this time. Isolation of these strains should be one of the main priorities for further research. It would also be interesting to see whether the ineffective *Frankia* nodulation capacity of the soil at location Oostvoorne (VD7) will increase when the average soil water-table will rise again, and how these strains are vertically distributed in the soil. Effective strains at location Oostvoorne were found in more or less equal numbers in wet and dryer soils at this location (Van Dijk and Sluimer-Stolk 1990). In the wet soils, competition between effective and ineffective *Frankia* could be expected. Due to the phenomenon of host- plant resistance to ineffective *Frankia* nodulation, the ineffective *Frankia* strains are unlikely to win the competition with effective strains for symbiotic growth. Consequently, they cannot increase their soil population due to natural nodule die-off (Van Dijk 1984) to the extent effective *Frankia* can do. Since the ineffective *Frankia* strains are nonetheless co-existing with effective *Frankia* strains in the same soil samples, and in some cases numerically dominant, this should mean that these ineffective strains are better equipped to live in a free-living mode within these waterlogged soils than these local effective

Frankia strains. The lack of success we experienced in isolating ineffective strains may seem to contradict this statement, but it may well be these particular *Frankia* have special growth requirements yet to be discovered.

Do ineffective Frankia *strains occur at (waterlogged) locations without* Alnus glutinosa*?* While ineffective *Frankia* strains do not seem to be dependent on nodulation of *A. glutinosa* for maintenance of their soil population, they may still require the alder as a source of specific root exudates, decomposing leaf litter, or some other growth factor. If this is the case, they should not be encountered in areas totally devoid of *A. glutinosa*. The fact they were found in high numbers in the rhizosphere of a *Salix* vegetation (see chapter 2b) could have partially been caused by run-off from the alder vegetation zone, which was located at a slightly higher elevation. Also, it should be investigated whether other actinorhizal plant species support ineffective *Frankia* soil populations.

The detection of alder-infective ineffective *Frankia* in soil is, at the moment, dependent on the bio-test assay, using clones of *A. glutinosa* plants, susceptible to ineffective *Frankia* nodulation. The use of clones will remain to be important for the study of *A. glutinosa* populations in the field but might in the near future be replaced by PCR amplification or *in situ* DNA hybridisation techniques for the purpose of detecting ineffective *Frankia* strains in soil.

With respect to the phylogeny and the physiology of ineffective nodule development of ineffective *Frankia* strains much remains to be studied. The fact that the non-isolated ineffective *Frankia* strains form a monophyletic cluster, related to effective *Frankia* strains presents some evolutionary questions. Guan *et al.* (1996) found that ineffective nodules contain high amounts of phenolic compounds. It was determined that certain nodule-specific genes, encountered in effective nodules, were absent in ineffective nodules on *A. glutinosa*. The research of the plant-endophyte interactions on the gene level will probably be one of the key instruments for furthering our understanding of this group of ineffective *Frankia* strains.

REFERENCES

Abeysekera R. M., Newcomb W., Silvester W. B. and Torrey J. G. (1989) A freeze-fracture electron microscopic study of *Frankia* in root nodules of *Alnus incana* grown at three oxygen tensions. *Canadian Journal of Microbiology* **36**, 97-108.

Akkermans A. D. L. (1971) Nitrogen fixation and nodulation of *Alnus* and *Hippophaë* under natural conditions. PhD thesis, Leiden State University, The Netherlands.

Akkermans A. D. L. and Hahn D. (1990) *Frankia* as a limiting factor in the growth of *Alnus* spp.: a critical evaluation, 331-336. In: D. Werner and P. Müller (ed.) *Fast growing trees and nitrogen fixing trees*. Proceedings International Conference, Marburg. Gustav Fischer Verlag, Stuttgart.

Akkermans A. D. L., Hahn D. and Mirza M. S. (1991) Molecular ecology of *Frankia*: Advantages and disadvantages of the use of DNA probes. *Plant and Soil* **137**, 49-54.

Akkermans A. D. L., Hahn D., and Zoon F. (1989) Interactions between root symbionts, root pathogens and actinorhizal plants. *Annales des Sciences Forestières* **46** (Suppl.), 765-771.

Akkermans A. D. L. and Hirsch A. M. (1997) A reconsideration of terminology in *Frankia* research: A need for congruence. *Physiologia Plantarum* **99**, 574-578.

Akkermans A. D. L., Huss-Danell K. and Roelofsen W. (1981) Enzymes of the tricarboxylic acid cycle and the malate-aspartate shuttle in the N_2-fixing endophyte of *Alnus glutinosa*. *Physiologia Plantarum* **53**, 289-294.

Akkermans A. D. L., Roelofsen W., Blom J., Huss-Danell K. and Harkink R. (1983) Utilization of carbon and nitrogen compounds by *Frankia* in synthetic media and in root nodules of *Alnus glutinosa*, *Hippophaë rhamnoides*, and *Datisca cannabina*. *Canadian Journal of Botany* **61**, 2793-2800.

Akkermans A. D. L. and Van Dijk C. (1976) The formation and nitrogen-fixing activity of the root nodules of *Alnus glutinosa* under field conditions, 511-521. In: P.S. Nutman (ed.) *Symbiotic Nitrogen Fixation in Plants*. Cambridge University Press, Cambridge.

Akkermans A. D. L. and Van Dijk C. (1981) Non-leguminous root-nodule symbioses with actinomycetes and *Rhizobium*, 57-103. In: W. J. Broughton (ed.) *Nitrogen Fixation.* Volume 1: Ecology. Clarendon Press, Oxford.

Allen M. B. and Arnon D. I. (1955) Studies on nitrogen fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. *Plant Physiology* **30**, 366-372.

Allen J. D., Silvester W. B. and Kalin M. (1966) *Streptomyces* associated with root nodules of *Coriaria* in New Zealand. *New Zealand Journal of Botany* **4**, 57-65.

Amarger N. (1981) Competition for nodule formation between effective and ineffective strains of *Rhizobium meliloti*. *Soil Biology and Biochemistry* **13**, 475-480.

Angle J. S. (1986) Pectic and proteolytic enzymes produced by fast- and slow-growing soybean rhizobia. *Soil Biology and Biochemistry* **18**, 115-116.

References

Angulo Carmona A. F. (1974) La formation des nodules fixatures d'azôte chez *Alnus glutinosa* (L.) Vill. *Acta Botanica Neerlandia* **23**, 257-303.

Angulo A. F., Van Dijk C. and Quispel A. (1975) Symbiotic interactions in non-leguminous root nodules, 475-483. In: P. S. Nutman (ed.) *Symbiotic Nitrogen Fixation in Plants*. Cambridge University Press, Cambridge.

Arveby A. S. and Huss-Danell K. (1988) Presence and dispersal of infective *Frankia* in peat and meadow soils in Sweden. *Biological Fertilisation of Soils* **6**, 39-44.

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

Baker D. D., Newcomb W. and Torrey J. G. (1980) Characterization of an ineffective actinorhizal microsymbiont, *Frankia* sp. EuI1 (Actinomycetales). *Canadian Journal of Microbiology* **26**, 1072-1089.

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

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

Baker D. D. and Torrey J. G. (1979) The isolation and cultivation of actinomycetous root nodule endophytes, 38-56. In: J. C. Gordon, C. T. Wheeler and D. A. Perry (ed.) *Symbiotic Nitrogen Fixation in the Management of Temperate Forests*. Forest Research Laboratory, Oregon State University, Corvallis.

Baker D. D. and Torrey J. G. (1980) Characterization of an effective actinorhizal microsymbiont, *Frankia* sp. AvcI1 (Actinomycetales). *Canadian Journal of Microbiology* **26**, 1066-1071.

Baker D. D., Torrey J. G. and Kidd G. H. (1979) Isolation by sucrose-density fractionation and cultivation *in vitro* of actinomycetes from nitrogen-fixing root nodules. *Nature* **281**, 76-78.

Becking J. H. (1968) Nitrogen fixation by non-leguminous plants. *Nitrogen, Dutch Nitrogenous Fertilizer Review* **12**, 47-74.

Becking J. H. (1970) *Frankiaceae* fam. nov. (*Actinomycetales*) with one new combination and six new species of the genus *Frankia* Brunchorst 1886, 174. *International Journal of Systematic Bacteriology* **20**, 201-220.

Becking J. H., De Boer W. E. and Houwink A. L. (1964) Electron microscopy of the endophyte of *Alnus glutinosa*. *Antonie van Leeuwenhoek* **30**, 343-376.

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

Benson D. R. (1982) Isolation of *Frankia* strains from alder actinorhizal root nodules. *Applied and Environmental Microbiology* **44**, 461-465.

Benson D. R., Buchholz S. E. and Hanna D. G. (1984) Identification of *Frankia* strains by two-dimensional polyacrylamide gel electrophoresis. *Applied and Environmental Microbiology* **47**, 489-494.

Benson D. R. and Hanna D. G. (1983) *Frankia* diversity in an alder stand as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell proteins. *Canadian Journal of Botany*, **61**, 2919-2923.

Benson D. R., Mazzuco C. E. and Browning T. J. (1985) Physiological Aspects of *Frankia*. In: P. W. Ludden and J. E. Burris (ed.) *Nitrogen Fixation and CO_2 Metabolism*. Elsevier Science B. V., Amsterdam.

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

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

Berg R. H., Liu L., Dawson J. O., Savka M. A. and Farrand S. K. (1992) Induction of pseudoactinorhizae by the plant pathogen *Agrobacterium rhizogenes*. *Plant Physiology* **98**, 777-779.

Berg R. H. and McDowell L. (1987) Endophyte differentiation in *Casuarina* actinorhizae. *Protoplasma* **136**, 104-117.

Bermúdez de Castro F., Míguel C. and Rodríguez-Barrueco C. (1976) A study of the capacity of soils to induce nodules in *Alnus glutinosa* (L.) Gaertn. and *Myrica gale* L., with special reference to the specificity of the endophytes. *Annales of Microbiology* **127** A, 307-315.

Berry A. M. (1984) The actinorhizal infection process: Review of recent research, 223-229. In: M. J. Klug and Reddy (ed.) *Current Perspectives in Microbial Ecology*, American Society of Microbiology Press, Washington DC.

Berry A. M., Kahn R. K. S. and Booth M. C. (1989) Identification of indole compounds secreted by *Frankia* HFPArI3 in defined culture medium. *Plant and Soil* **118**, 205-209.

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

Berry A. M. and Sunell L. A. (1990) The infection process and nodule development. In: C. R. Schwintzer and J. D. Tjepkema (ed.) *The Biology of Frankia and Actinorhizal Plants*, 61-81, Academic Press, New York.

Berry A. M. and Torrey J. G. (1979) Isolation and characterization in vivo and in vitro of an actinomycetous endophyte from *Alnus rubra* Bong., 69-83. In: J. C. Gordon, C. T. Wheeler and D. A. Perry (ed.) *Symbiotic Nitrogen Fixation in the Management of Temperate Forests*. Forest Research Laboratory, Oregon State University, Corvallis.

Berry A. M. and Torrey J. G. (1983) Root hair deformation in the infection process of *Alnus rubra*. *Canadian Journal of Botany* **61**, 2863-2876.

Binkley D., Lousier J. D. and Cromack K., Jr (1984) Ecosystem effects of sitka alder in a douglas-fir plantation. *Forest Science* **30**, 26-35.

References

Blom J. (1981) Utilization of fatty acids and NH_4^+ by *Frankia* AvcI1. *FEMS Microbiology Letters* **10**, 143-145.

Blom J., Roelofsen W. and Akkermans A. D. L. (1981) Assimilation of nitrogen in root nodules of alder (*Alnus glutinosa*). *New Phytologist* **89**, 321-326.

Blom J. (1982) Carbon and nitrogen metabolism of free-living *Frankia* spp. and of *Frankia-Alnus* symbioses. PhD thesis, Wageningen Agricultural University, The Netherlands.

Bohlool B. B. and Schmidt E. L. (1974) Lectins: A possible base for specificity in the *Rhizobium*-legume root nodule symbiosis. *Science* **185**, 269-271.

Bollen W. B., Chen C., Lu K. C. and Tarrant R. F. (1967) Influence of red alder on fertility of a forest soil - Microbial and chemical effects. *Research Bulletin* **12** of National Science Foundation, grant 21015.

Bond (1959) Fixation of nitrogen in non-legume root-nodule plants. *Symposia of the Society for Experimental Biology* **XIII**, Utilization of Nitrogen and its Compounds by Plants, 59-72.

Bond G. (1977) Some reflections on *Alnus*-type root nodules, 531-537. In: W. E. Newton, J. R. Postgate and C. Rodríguez-Barrueco (ed.) *Recent Developments in Nitrogen Fixation*, Academic Press, London.

Bond G. (1983) Taxonomy and distribution of non-legume nitrogen-fixing systems, 55-87. In: J. C. Gordon and C. T. Wheeler (ed.) *Biological nitrogen fixations in forest ecosystems: foundations and applications*. Martinus Nijhoff / Dr W. Junk Publishers, The Hague.

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

Bousquet J., Cheliak W. M. and Lalonde M. (1986) Allozyme variability in natural populations of green alder (*Alnus crispa*) in Quebec. *Genome* **29**, 345-352.

Bousquet J., Girouard E., Strobeck C., Dancik B. P. and Lalonde M. (1989) Restriction fragment polymorphisms in the rDNA region among seven species of *Alnus* and *Betula papyrifera*. *Plant and Soil* **118**, 231-240.

Britten R. J. (1986) Rates of DNA sequence evolution differ between taxonomic groups. *Science* **231**, 1393-1398.

Burggraaf A. J. P., Quispel A., Tak T. and Valstar J. (1981) Methods of isolation and cultivation of *Frankia* species from actinorhizas. *Plant and Soil* **61**, 157-168.

Burggraaf A. J. P. and Shipton W. A. (1982) Estimates of *Frankia* growth under various pH and temperature regimes. *Plant and Soil* **69**, 135-147.

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

Caetano-Anollés G. and Gresshoff P. M. (1991) Excision of nodules induced by *Rhizobium meliloti* exopolysaccharide mutants releases autoregulation in alfalfa. *Journal of Plant Physiology* **138**, 765-767.

Callaham D., Del Tredici P. and Torrey J. G. (1978) Isolation and cultivation *in vitro* of the actinomycete causing root nodulation in *Comptonia*. *Science* **199**, 899-902.

Callaham D., Newcomb W., Torrey J.G. and Peterson R. L. (1979) Root hair infection in actinomycete-induced root initiation in *Casuarina*, *Myrica*, and *Comptonia*. *Botanical Gazette* **140** (Suppl.), S1-S9.

Capellano A., DeQuatre B., Valla G. and Moiroud A. (1987) Root-nodules formation by *Penicillium* sp. on *Alnus glutinosa* and *Alnus incana*. *Plant and Soil* **104**, 45-51.

Carú M. (1993) Characterization of native *Frankia* strains isolated from Chilean shrubs (Rhamnaceae). *Plant and Soil* **157**, 137-145.

Carpenter P. L. and Hensley D. L. (1979) Utilizing N_2-fixing woody plant species for distressed soils and the effect of lime on survival. *Botanical Gazette* **140** (Suppl.), S76-S81.

Cavelli-Sforza L. L. and Edwards A. W. F. (1967) Phylogenetic analysis: models and estimation procedures. *American Journal of Human Genetics* **19**, 233-257.

Chaboud A. and Lalonde M. (1983) Lectin binding on surfaces of *Frankia* strains. *Canadian Journal of Botany* **61**, 2889-2897.

Chase M., Soltis D. E., Olmstead R. G., Morgan D., Les D. H., Mishler B. D., Duvall M. R., Price R., Hills H. G., Qiu Y., Kron K. A., Rettig J. H., Conti E., Palmer J. D., Manhart J. R., Sytsma K. J., Michaels H. J., Kress W. J., Donoghue J. D., Clark W. D., Hedrén M., Gaut B. S., Jansen R. K., Kim K.-J., Wimpee C. F., Smith J. F., Furnier G. R., Straus S. H., Xiang Q., Plunkett G. M., Soltis P. S., Swensen S. M., Eguiarte L. E., Learn G. H. Jr., Barrett S. C., Graham S. and Albert V. A. (1993) Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene *rbc*L. *Annals of the Missouri Botanical Garden* **80**, 528-580.

Chatarpaul L., Chakravarty P. and Subramaniam P. (1989) Studies in tetrapartite symbioses. I. *Role of ecto- and endomycorrhizal fungi and* Frankia *on the growth performance of* Alnus incana. *Plant and Soil* **118**, 145-150.

Chaudhary A. H. and Mirza M. S. (1987) Isolation and characterization of *Frankia* from nodules of actinorhizal plants of Pakistan. *Physiologia Plantarum* **70**, 255-258.

Church G. M. and Gilbert W. (1984) Genomic sequencing. *Proceedings of the National Academy of Sciences of the USA* **81**, 1991-1995.

Dawson J. O. (1986) Actinorhizal plants: their use in forestry and agriculture. *Outlook on Agriculture* **15**, 202-208.

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

Diem H. G., Gauthier D. and Dommergues Y. R. (1982) Isolation of *Frankia* from nodules of *Casuarina equisetifolia*. *Canadian Journal of Microbiology* **28**, 526-530.

References

Diem H. G., Gauthier D. and Dommergues Y. R. (1983) An effective strain of *Frankia* from *Casuarina* sp. *Canadian Journal of Botany* **61**, 2815-2821.

Djordjevic M. A., Gabriel D. W. and Rolfe B. G. (1987) *Rhizobium*, the refined parasite of legumes. *Annual Reviews of Phytopathology* **25**, 145-168.

Dobritsa S. V. (1985) Restriction analysis of the *Frankia* spp. genome. *FEMS Microbiology Letters* **29**, 123-128.

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

Dobritsa S. V. and Stupar O. S. (1989) Genetic heterogeneity among *Frankia* isolates from root nodules of individual actinorhizal plants. *FEMS Microbiologial Letters* **58**, 287-292.

Domenach A. M., Kurdali F. and Bardin R. (1989) Estimation of symbiotic dinitrogen fixation in alder forest by the method based on natural ^{15}N abundance. *Plant and Soil* **118**, 51-59.

Edwards A. W. F. and Cavalli-Sforza L. L. (1963) The reconstruction of evolution. *Heredity* **18**, 553.

Embley T. M., Smida J. and Stackebrandt E. (1988) Reverse transcriptase sequencing of 16S ribosomal RNA from *Faenia rectivirgula*, *Pseudonocardia thermophyla* and *Saccharopolyspora hirsuta*, three wall type IV actinomycetes which lack mycolic acids. *Journal of General Microbiology* **134**, 961-966.

Faure-Raynaud M., Daniere C., Moiroud A. and Capellano A. (1991) Diversity of *Frankia* strains isolated from single nodules of *Alnus glutinosa*. *Plant and Soil* **132**, 207-211.

Felsenstein (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution* **17**, 368-376.

Felsenstein J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**, 783-791.

Felsenstein J. (1988) Phylogenies from molecular sequences: Inference and reliability. *Annual Reviews of Genetics* **22**, 521-565.

Felsenstein J. (1989) PHYLIP -- Phylogeny Inference Package (Version 3.2). *Cladistics* **5**, 164-166.

Fernandez M. P., Meugnier H., Grimont P. A. D. and Bardin R. (1989) Deoxyribonucleic acid relatedness among members of the genus *Frankia*. *International Journal of Systematic Bacteriology* **39**, 424-429.

Fisher R. F. and Long S. R. (1992) *Rhizobium*-plant signal exchange. *Nature* **357**, 655-660.

Fitch W. M. and Margoliash E. (1967) Construction of phylogenetic trees. *Science* **155**, 279-284.

Gardes M., Bousquet J. and Lalonde M. (1987) Isozyme variation among 40 *Frankia* strains. *Applied and Environmental Microbiology* **53**, 1596-1603.

Gardes M. and Lalonde M. (1987) Identification and subgrouping of *Frankia* strains using sodium dodecyl sulfate polyacrylamide gel electrophoresis. *Physiologia Plantarum* **70**, 237-244.

Gardner I. C. and Scott A. (1982) The effect of altered habitat on nitrogen metabolism in some free-living and symbiotic relationships involving nitrogen fixing organisms. *Chemistry in Ecology* **1**, 47-55.

Gardner I. C., Clelland D. M. and Scott A. (1984) Mycorrhizal improvement in non-leguminous nitrogen fixing associations with particular reference to *Hippophaë rhamnoides* L. *Plant and Soil* **78**, 189-199.

Gatz C. (1997) Chemical control of gene expression. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**, 89-108.

Gauthier D., Frioni L., Diem H. G. and Dommergues Y. (1984) The *Colletia spinosissima-Frankia* symbiosis. *Acta Œcologica* **5**, 231-239.

Gherbi H., Duhoux E., Franche C., Pawlowski K., Nasser A., Berry A. M. and Bogusz D. (1997) Cloning of a full-length symbiotic hemoglobin cDNA and *in situ* localization of the corresponding mRNA in *Casuarina glauca* root nodule. *Physiologia Plantarum* **99**, 608-616.

Girgis M. G. Z., Ishac Y. Z., Diem H. G. and Dommergues Y. R. (1992) Selection of salt tolerant *Casuarina glauca* and *Frankia*. *Acta Œcologia* **13**, 443-451.

Granhall U., Ericsson T. and Clarholm M. (1983) Dinitrogen fixation and nodulation by *Frankia* in *Alnus incana* as affected by inorganic nitrogen in pot experiments with peat. *Canadian Journal of Botany* **61**, 2856-2963.

Guan C. (1997) Plant gene expression in actinorhizal nodules of *Alnus glutinosa*. PhD thesis, Wageningen Agricultural University, The Netherlands.

Guan C., Akkermans A. D. L., Van Kammen A., Bisseling T. and Pawlowski K. (1997) *ag* 13 is expressed in *Alnus glutinosa* nodules in infected cells during endosymbiont degradation and in the nodule pericycle. *Physiologia Plantarum* **99**, 601-607.

Guan C., Wolters D. J, Van Dijk C., Akkermans A. D. L., Van Kammen A., Bisseling T. and Pawlowski K. (1996) Gene expression in ineffective actinorhizal nodules of *Alnus glutinosa*. *Acta Botanica Gallica* **143**, 613-620.

Hafeez F., Akkermans A. D. L. and Chaudhary A. H. (1984a) Morphology, physiology and infectivity of two *Frankia* isolates An 1 and An 2 from root nodules of *Alnus nitida*. *Plant and Soil* **78**, 45-59.

Hafeez F., Chaudhary A. H. and Akkermans A. D. L. (1984b) Physiological studies on N_2-fixing root nodules of *Datisca cannabina* L. and *Alnus nitida* Endl. from Himalaya region in Pakistan. *Plant and Soil* **78**, 129-146.

Hagedorn C. (1978) Effectiveness of *Rhizobium trifolii* populations associated with *Trifolium subterraneum* L. in southwest Oregon soils. *Soil Science Society of America Journal* **42**, 447-451.

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

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

References

Hahn D., Lechevalier M. P., Fischer A. and Stackebrandt E. (1989b) Evidence for a close phylogenetic relationship between members of the genera *Frankia*, *Geodermatophilus*, and "*Blastococcus*" and emendation of the family *Frankiaceae*. *Systematic and Applied Microbiology* **11**, 236-242.

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

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

Harris S. and Silvester W. (1992) Nitrogenase activity and growth of *Frankia* in batch and continuous culture. *Canadian Journal of Microbiology* **38**, 296-302.

Harry D. E., Yang D. C. and Dawson J. O. (1991) Nucleotide sequence and diversity in 16S ribosomal RNA from *Frankia*. *Plant and Soil* **131**, 143-146.

Head I. M., Saunders J. R. and Pickup R. W. (1998) Microbial evolution, diversity, and ecology: A decade of ribosomal RNA analysis of uncultivated microorganisms. *Microbial Ecology* **35**, 1-21.

Heath M. C. (1991) Tansley Review No. 33: Evolution of resistance to fungal parasitism in natural ecosystems. *New Phytologist* **119**, 331-343.

Hendrickson O. Q., Burgess D., Perinet P., Tremblay F. and Chatatpaul L. (1993) Effects of *Frankia* on field performance of *Alnus* clones and seedlings. *Plant and Soil* **150**, 295-302.

Hennessey T. C., Vishniac H. S., Lorenzi E. M. and Williams J. C. (1989) Dinitrogen fixation in a water-stressed *Alnus* clone is limited by host xerotolerance. *Plant and Soil* **118**, 89-96.

Higgins, D. G., Bleasby A. J. and Fuchs R. (1992) CLUSTAL V: improved software for multiple sequence alignment. *Computer Applications in the Biosciences* **8**, 189-191.

Hillis D. M. (1997) Biology recapitulates phylogeny. *Science* **276**, 218-291.

Hillis D. M., Huelsenbeck J. P. and Cunningham C. W. (1994) Application and accuracy of molecular phylogenies. *Science* **264**, 671-677.

Holman R. M. and Schwintzer C. R. (1987) Distribution of spore-positive and spore-negative nodules of *Alnus incana* subsp. *rugosa* in Maine, USA. *Plant and Soil* **104**, 103-111.

Hönerlage W., Hahn D., Zepp K., Zeyer J. and Normand P. (1994) A hypervariable 3S rRNA region provides a discriminating target for specific characterization of uncultured and cultured *Frankia*. *Systematic and Applied Microbiology* **17**, 433-443.

Hooker J. E. and Wheeler C. T. (1987) The effectivity of *Frankia* for nodulation and nitrogen fixation in *Alnus rubra* and *A. glutinosa*. *Physiologia Plantarum* **70**, 333-341.

Horrière F. (1984) *In vitro* physiological approach to classification of *Frankia* isolates of 'the Alnus group', based on urease, protease, and ß-glucosidase activities. *Plant and Soil* **78**, 7-13.

Houwers A. and Akkermans A. D. L. (1981) Influence of inoculation on yield of *Alnus glutinosa* in the Netherlands. *Plant and Soil* **61**, 189-202.

Huss-Danell K., Lundquist P-O. and Ekblad A. (1989) Growth and acetylene reduction activity by intact plants of *Alnus incana* under field conditions. *Plant and Soil* **118**, 61-73.

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

Huss-Danell K., Roelofsen W., Akkermans A. D. L. and Meijer P. (1982a) Carbon metabolism of *Frankia* spp. in root nodules of *Alnus glutinosa* and *Hippophaë rhamnoides*. *Physiologia Plantarum* **54**, 461-466.

Huss-Danell K., Sellstedt A., Flower-Ellis A. and Sjöström M. (1982b) Ammonium effects on function and structure of nitrogen-fixing root nodules of *Alnus incana* (L.) Moench. *Planta* **156**, 332-340.

Huss-Danell K., Winship L. J. and Hahlin A. (1987) Loss and recovery of nitrogenase in *Alnus incana* nodules exposed to low oxygen and low temperature. *Physiologia Plantarum* **70**, 355-360.

Ishizuka J. (1992) Trends in biological nitrogen fixation research and application. *Plant and Soil* **141**, 197-209.

Jamann S., Fernandez M. P. and Moiroud A. (1992) Genetic diversity of Elaeagnaceae-infective *Frankia* strains isolated from various soils. *Acta Œcologia* **13**, 395-405.

Jin L. and Nei M. (1990) Limitations of the evolutionary parsimony method of phylogenetic analysis. *Molecular Biological Evolution* **7**, 82-102.

Jones O. P., Hopgood M. E. and O'Farrell (1977) Propagation *in vitro* of M.26 apple rootstocks. *Journal of Horticultural Science* **52**, 235-238.

Jukes T. H. and Cantor C. R. (1969) Evolution of protein molecules. In: H. N. Munro (ed.) *Mammalian Protein Metabolism*. Academic Press, New York.

Karban R., Agrawal A. A. and Mangel M. (1997) The benefits of induced defenses against herbivores. *Ecology* **78**, 1351-1355.

Kashanski C. R. and Schwintzer C. R. (1987) Distribution of spore-positive and spore-negative nodules of *Myrica gale* in Maine, USA. *Plant and Soil* **104**, 113-120.

Kimura M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**, 111-120.

Knowlton S., Berry A. and Torrey J. G. (1980) Evidence that associated soil bacteria may influence root hair infection of actinorhizal plants by *Frankia*. *Canadian Journal of Microbiology* **26**, 971-977.

Knowlton S. and Dawson J. O. (1982) Effects of *Pseudomonas cepacia* and cultural factors on the nodulation of *Alnus rubra* roots by *Frankia*. *Canadian Journal of Botany* **61**, 2877-2882.

Knowlton S. and Dawson J. O. (1983) Effects of *Pseudomonas cepacia* and cultural factors on the nodulation of *Alnus rubra* roots by *Frankia*. *Canadian Journal of Microbiology* **26**, 971-977.

References

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

Kurdali F., Capellano, A., Moiroud A. and Domenach A. M. (1989) Study of the contribution of the shoot and/or root of *Alnus* sp. in the compatibility between the host and a Sp+ *Frankia* strain using a grafting technique. *Plant and Soil* **113**, 101-109.

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

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

Lalonde M. and Calvert H. E. (1979) Production of *Frankia* hyphae and spores as an infective inoculant for *Alnus* species, 95-110. In: J. C. Gordon, C. T.Wheeler and D. A. Perry (ed.). *Symbiotic nitrogen fixation in the management of temperate forests*. Forest Research Laboratory, Oregon State University, Corvallis.

Lalonde M. and Knowles R. (1975) Ultrastructure, composition and biogenesis of the encapsulation material surrounding the endophyte in *Alnus crispa* var. *mollis* root nodules. *Canadian Journal of Botany* **53**, 1951-1971.

Lalonde M., Simon L., Bousquet J. and Séguin A. (1988) Advances in the taxonomy of *Frankia*: Recognition of species *Alni* and *Elaeagni* and novel subspecies *Pommerii* and *Vandijkii*. In: H. Bothe, F. J. DeBruijn and W. E. Newton (ed.) *Nitrogen fixation: hundred years after*. Gustav Fischer, New York.

Lawrence D. B., Schoenike R. E., Quispel A. and Bond G. (1967) The role of *Dryas drummondii* in vegetation development following ice recession at Glacier Bay, Alaska, with special reference to its nitrogen fixation by root rodules. *Journal of Ecology* **55**, 793-813.

Lechevalier M. P. (1983) Cataloguing *Frankia* strains. *Canadian Journal of Botany* **61**, 2964-2967.

Lechevalier M. P., Baker D. and Horrière F. (1983) Physiology, chemistry, serology, and infectivity of two *Frankia* isolates from *Alnus incana* subsp. *rugosa*. *Canadian Journal of Botany* **61**, 2826-2833.

Lechevalier M. P. and Lechevalier H. A. (1979) The taxonomic position of the actinomycetic endophytes. In: J.C. Gordon, C. T. Wheeler and D. A. Perry (ed.) *Symbiotic Nitrogen Fixation in the Management of Temperate Forests*. Forest Research Laboratory, Oregon State University, Corvallis.

Lechevalier M. P. and Ruan J. S. (1984) Physiology and chemical diversity of *Frankia* spp. isolated from nodules of *Comptonia peregrina* (L.) Coult. and *Ceanothus americanus* (L.). *Plant and Soil* **78**, 15-22.

Lipman D. J. and Pearson W. R. (1985) Rapid and sensitive protein similarity searches. *Science* **227**, 1435-1441.

Ludwig W. (1995) Sequence Databases, Chapter 3.3.5: 1-22. In: A. D. L. Akkemans, J. D. van Elsas and F. J. de Bruijn (ed.) *Molecular Microbial Ecology Manual*. Kluwer Academic Publishers, Dordrecht, The Netherlands.

MacConnel J. T. (1959) The oxygen factor in the development and function of the root nodules of alder. *Annals of Botany*, N.S. **23**, 261-269.

MacConnel J. T. and Bond G. (1957) A comparison of the effect of combined nitrogen on nodulation of in non-legumes and legumes. *Plant and Soil* **8**, 378-388.

MacKay J., Simon L. and Lalonde M. (1987) Effect of substrate nitrogen on the performance of *in vitro* propagated *Alnus glutinosa* clones inoculated with Sp^+ and Sp^- *Frankia* strains. *Plant and Soil* **103**, 21-31.

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

Maidak B. L., Olsen G. J., Larsen N. *et al.* (1996) The Ribosomal Database Project (RDP). *Nucleic Acids Research* **24**, 82-85.

Maniatis T., Fritsch E. F. and Sambrook J. (1982) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, New York.

Mauricio R., Rausher M. D. and Burdick D. S. (1997) Variation in the defense strategies of plants: are resistance and tolerance mutually exclusive? *Ecology* **78**, 1301-1311.

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

Meesters T. M., Van Vliet W. M. and Akkermans A. D. L. (1987) Nitrogenase is restricted to the vesicles in *Frankia* strain EAN1pec. *Physiologia Plantarum* **70**, 267-271.

Mian S. and Bond G. (1976) Effective and ineffective root nodules in *Myrica faya*. *Proceedings of the Royal Society of London Botanists* **194**, 285-293.

Mian S. and Bond G. (1978) The onset of nitrogen fixation in young alder plants and its relation to differentiation in the nodular endophyte. *New Physiology* **80**, 187-192.

Míguel C., Cañizo A., Costa A. and Rodríguez-Barrueco C. (1978) Some aspects of the *Alnus*-type root nodule symbiosis, 121-133. In: J. Döbereiner, R. H. Burris, Holländer *et al*. (ed.) *Limitations and Potentials for Biological Nitrogen Fixation in the Tropics*, 121-133, Plenum Press, New York.

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

Miller I. M. and Baker D. D. (1986) Nodulation of actinorhizal plants by *Frankia* strains capable of both root hair infection and intercellular penetration. *Protoplasma* **131**, 82-91.

Mindell D. P. (1991) Aligning DNA sequences: Homology and phylogenetic weighting (Chapter 5), 73-89. In: M. M. Miyamoto and J. Cracraft (ed.) *Phylogenetic Analysis of DNA Sequences*. Oxford University Press, New York.

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

References

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

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

Mirza M. S., Janse J. D., Hahn D. and Akkermans A. D. L. (1991) Identification of atypical *Frankia* strains by fatty acid analysis. *FEMS Microbiology Letters* **83**, 91-98.

Miyamoto M. M. and Cracraft J. (ed.) (1994) *Phylogenetic Analysis of DNA sequences*. Oxford University Press, New York.

Monz C. A. and Schwintzer C. R. (1989) The physiology of spore-negative and spore-positive nodules of *Myrica gale*. *Plant and Soil* **118**, 75-87.

Mullin B. C. and Dobritsa S. V. (1996) Molecular analysis of actinorhizal symbiotic systems: Progress to date. *Plant and Soil* **186**, 9-20.

Murashige T and Skoog F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497.

Murry M. A., Fontaine M. S. and Tjepkema J. D. (1984) Oxygen protection of nitrogenase in *Frankia* sp. HFPArI3. *Archives of Microbiology* **139**, 162-166.

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

Murry M. A., Zhongze Z. and Torrey J. G. (1985) Effect of O_2 on vesicle formation, acetylene reduction, and O_2-uptake kinetics in *Frankia* sp. HFPCcI3 isolated from *Casuarina cunninghamiana*. *Canadian Journal of Microbiology* **31**, 804-809.

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

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

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

Nazaret S., Simonet P., Normand P. and Bardin R. (1989) Genetic diversity among *Frankia* isolated from *Casuarina* nodules. *Plant and Soil* **118**, 241-247.

Nei M. (1991) Relative efficiencies of different tree-making methods for molecular data (Chapter 6), 90-128. In: M. M. Miyamoto and J. Cracraft (ed.) *Phylogenetic Analysis of DNA Sequences*. Oxford University Press, New York.

Nei M. (1997) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

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

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

Nittayajarn A., Mullin B. C. and Baker D. D. (1990) Screening of symbiotic *Frankiae* for host specificity by restriction fragment length polymorphism analysis. *Applied and Environmental Microbiology* **56**, 1172-1174.

Noridge N. A. and Benson D. R. (1986) Isolation and nitrogen-fixing activity of *Frankia* sp. strain CpI1 vesicles. *Journal of Bacteriology* **166**, 301-305.

Normand P. and Lalonde M. (1982) Evaluation of *Frankia* strains isolated from provenances of two *Alnus* species. *Canadian Journal of Microbiology* **28**, 1133-1142.

Normand P. and Lalonde M. (1986) The genetics of actinorhizal *Frankia*: A review. *Plant and Soil* **90**, 429-453.

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

Parsons R., Silvester W. B., Harris S., Gruijters W. T. M. and Bullivant S. (1987) *Frankia* vesicles provide inducible and absolute oxygen protection for nitrogenase. *Plant Physiology* **83**, 728-731.

Paschke M. W. (1997) Actinorhizal plants in rangelands of the Western United States. *Journal of Range Management* **50**, 62-72.

Paschke M. W. and Dawson J. O. (1990) *Frankia* infective capacity is enhanced in a *Betula nigra* plantation soil. *Forestry Research Report* **90**, Agricultural Experiment Station, University of Illinois, Urbana-Champaign.

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

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

Paschke M. W., Dawson J. O. and David M. B. (1989) Soil nitrogen mineralization in plantations of *Juglans nigra* interplanted with actinorhizal *Elaeagnus umbellata* or *Alnus glutinosa*. *Plant and Soil* **118**, 33-42.

Pawlowski K. (1997) Nodule-specific gene expression. *Physiologia Plantarum* **99**, 617-631.

Pawlowski K., Akkermans A. D. L., Van Kammen A. and Bisseling T. (1995) Expression of *Frankia nif* genes in nodules of *Alnus glutinosa*. *Plant and Soil* **170**, 371-376.

Pawlowski K., Ribeiro A. and Bisseling T. (1996) Nitrogen fixing root nodule symbioses: legume nodules and actinorhizal nodules. In: M. R. El-Gewely (ed.) *Biotechnology Annual Review* **2**, 151-184, Elsevier Science B. V., Amsterdam, The Netherlands.

References

Périnet P. and Lalonde M. (1983) *In vitro* propagation and nodulation of the actinorhizal host plant *Alnus glutinosa* (L.) Gaertn. *Plant Science Letters* **29**, 9-17.

Périnet P., Vallée G. and Tremblay F. M. (1988) In vitro propagation of mature trees of *Alnus incana* (L.) Moench. *Plant Cell, Tissue and Organ Culture* **15**, 85-89.

Perradin Y., Mottet M. J. and Lalonde M. (1983) Influence of phenolics on *in vitro* growth of *Frankia* strains. *Canadian Journal of Botany* **61**, 2807-2814.

Peters N. K., Frost J. W. and Long S. R. (1986) A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. *Science* **233**, 977-980.

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

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

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

Probanza A., Lucas J. A., Acero N. and Gutierrez Mañero F. J. (1996) The influence of native rhizobacteria on european alder (*Alnus glutinosa* (L.) Gaertn.) growth. *Plant and Soil* **182**, 59-66.

Promega Corp. (1994), pGEM®*-T Vector Systems, *Promega Technical Bulletin* **150**, 1-15.

Quispel A. (1954a) Symbiotic nitrogen fixation in non-leguminous plants. I. Preliminary experiments on the root-nodule symbiosis of *Alnus glutinosa* (L.) Gaertn. *Acta Botanica Neerlandica* **3**, 495-511.

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

Quispel A., Burggraaf A. J. P., Borsje H. and Tak T. (1983) The role of lipids in the growth of *Frankia* isolates. *Canadian Journal of Botany* **61**, 2801-2806.

Quispel A., Baerheim Svendsen A., Schripsema J., Baas W. J., Erkelen C. and Lugtenburg J. (1989) Identification of dipterocarpol as isolation factor for the induction of primary isolation of *Frankia* from root nodules of *Alnus glutinosa* (L.) Gaertner. *Molecular Plant-Microbe Interactions* **2**, 107-112.

Quispel A. and Tak T. (1978) Studies on the growth of the endophyte of *Alnus glutinosa* (L.) Vill. in nutrient solutions. *New Phytologist* **81**, 587-600.

Racette S. and Torrey J. G. (1989) The isolation, culture and infectivity of a *Frankia* strain from *Gymnostoma papuanum* (Casuarinaceae). *Plant and Soil* **118**, 165-170.

Ramírez-Saad H. C., Akkermans W. L. and Akkermans A. D. L. (1996) DNA extraction from actinorhizal nodules, 1-11. In: A. D. L. Akkermans, J. D. van Elzas and F. J. de Bruijn (ed.) *Molecular Microbial Ecology Manual.* Chapter 1.4.4. Kluwer Academic Publishers, Dordrecht, The Netherlands

Ramírez-Saad H. C., Janse J. D. and Akkermans A. D. L. (1998) Root nodules of *Ceanothus caeruleus* contain both the N_2-fixing *Frankia* endophyte and a phylogenetically related Nod^-/Fix^- actinomycete. *Canadian Journal of Microbiology* **44**, 140-148.

Reddell P. and Bowen G. D. (1985) Do single nodules of *Casuarinaceae* contain more than one *Frankia* strain? *Plant and Soil* **88**, 275-279.

Reddell P. and Spain A. V. (1991) Transmission of infective *Frankia* (Actinomycetales) propagules in casts of the endogeic earthworm *Pontoscolex corethrurus* (Oligochaeta: Glossoscolecidae). *Soil Biology and Biochemistry* **23**, 775-778.

Relic B., Perret X., Estrada-García M. T., Kopcinska J., Galinowski W., Krishnan H. B., Püppke S. G. and Broughton W. J. (1994) Nod factors of *Rhizobium* are a key to the legume door. *Molecular Microbiology* **13**, 171-178.

Ribeiro A. (1996) Plant genes involved in the establishment of an actinorhizal symbiosis. PhD thesis, Wageningen Agricultural University, The Netherlands.

Ribeiro A., Akkermans A. D. L., Van Kammen A., Bisseling T. and Pawlowski K. (1995) A nodule-specific gene encoding a subtilisin-like protease is expressed in early stages of actinorhizal nodule development. *The Plant Cell* **7**, 785-794.

Ringø E., Clausen E., Løvaas E., Van Ghelue M. and Solheim B. (1995) Effects of extracts of *Alnus glutinosa* seeds on growth of *Frankia* strain ArI3 under static and fermentor culture conditions. *Plant and Soil* **176**, 283-288.

Rogers R. D. and Wollum A. G. (1974) Virulence of the *Alnus* endophyte after *in vitro* cultivation. *Proceedings of the Soil Science Society of America* **38**, 756-759.

Rojas N. S., Perry D. A., Li C.Y. and Friedman J. (1992) Influence of actinomycetes on *Frankia* infection, nitrogenase activity and seedling growth of red alder. *Soil Biology and Biochemistry* **23**, 1043-1049.

Rönkkö R., Smolander A., Nurmiaho-Lassila E.-L. and Haahtela K. (1993) *Frankia* in the rhizosphere of nonhost plants: A comparison with root-associated N_2-fixing *Enterobacter*, *Klebsiella* and *Pseudomonas*. *Plant and Soil* **153**, 85-95.

Roy A. and Bousquet J. (1996) The evolution of the actinorhizal symbiosis through phylogenetic analysis of host plants. *Acta Botanica Gallica*, **143**, 635-650.

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

Safo-Sampah S. and Torrey J. G. (1988) Polysaccharide-hydrolyzing enzymes of *Frankia* (Actinomycetales). *Plant and Soil* **112**, 89-97.

Saitou R. R. and Nei M. (1987) A Neigbour-Joining method: a new method for reconstructing phylogenetic trees. *Molecular Biological Evolution* **44**, 406-425.

References

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

SAS/STAT(R) User's Guide, Version 6, Fourth Edition, Volumes 1 and 2, 1-1848, ISBN: 1-55544-376-1.

Savka M. A., Liu L., Farrand S. K., Berg R. H. and Dawson J. O. (1992) Induction of hairy roots or pseudoactinorhizae on *Alnus glutinosa*, *A. acuminata* and *Elaeagnus angustifolia* by *Agrobacterium rhizogenes*. *Acta Œcologia* **13**, 423-431.

Savouré A. and Lim G. (1991) Characterization of an effective *Frankia* (ISU 0224887) isolated from nodules of *Gymnostoma sumatranum*. *Plant and Soil* **131**, 21-27.

Sbay H., Guillot J. Danthu P. and Prat D. (1989) In vitro propagation of interspecific hybrids in *Alnus*. *Annales des Sciences Forestières* **46** (Suppl.), S155-S157.

Schwencke J. (1991) Rapid, exponential growth and increased biomass yield of some *Frankia* strains in buffered and stirred mineral medium (BAP) with phosphatidyl choline. *Plant and Soil* **137**, 37-41.

Schwencke J. (1994) Biochemical studies with *Frankia* – Problems and hopes, 245-252. In: M. Sadiki and A. Hilali (ed.) *Recent Developments in Biological Nitrogen Fixation Research in Africa*, LAV Hassan II.

Séguin A. and Lalonde M. (1989) Detection of pectinolytic activity and pel homologous sequences in *Frankia*. *Plant and Soil* **118**, 221-229.

Séguin A. and Lalonde M. (1990) Micropropagation , tissue culture , and genetic transformation of actinorhizal plants and *Betula*, 215-238. In: C. R. Schwintzer and J. D. Tjepkema (ed.) *The Biology of Frankia and Actinorhizal Plants*, Academic Press, New York.

Selim S., Delacour S. and Schwencke J. (1996) Specific long-chain fatty acids promote optimal growth of *Frankia*: accumulation and intracellular distribution of palmitic and propionic acid. *Archives of Microbiology* **165**, 252-257.

Selim S. and Schwencke J. (1994) 1,2-Dipalmitoyl phosphatidylcholine, 1,2-dipalmitoyl phosphatidic acid or 1,2-dipalmitoyl-*sn*-glycerol inhibit sporangia formation and promote exponential growth of various *Frankia* isolates from the Casuarinaceae family. *Soil Biology and Biochemistry* **26**, 569-575.

Sellstedt A. and Mattson U. (1994) Hydrogen metabolism in *Casuarina-Frankia*: Immunolocalization of nitrogenase and hydrogenase. *Soil Biology and Biochemistry* **26**, 583-592.

Sequerra J., Capellano A., Faure-Raynard M. and Moiroud A. (1994) Root hair infection process and myconodule formation on *Alnus incana* by *Penicillium nodositatum*. *Canadian Journal of Botany* **72**, 955-962.

Sequerra J., Capellano A., Gianinazzi-Pearson V. and Moiroud A. (1995) Ultrastructure of cortical root cells of *Alnus incana* infected by *Penicillium nodositatum*. *New Phytologist* **130**, 545-555.

Sequerra J., Marmeisse R., Valla G., Normand P., Capellano A. and Moiroud A. (1997) Taxonomic position and intraspecific variability of the nodule forming *Penicillium nodositatum* inferred from RFLP analysis of the ribosomal intergenic spacer and Random Amplified Polymorphic DNA. *Mycological Research* **101**, 465-472.

Sereno P. C., Wilson J. A., Larsson H. C. E., Dutheil D. B. and Sues H.-D. (1994) Early Cretaceous dinosaurs from the Sahara. *Science* **266**, 267-271.

Shantaram S., DuTeau N. M., Prakash R. K. and Atherly A. G. (1987) Comparative cytology of effective and ineffective root nodules of North American cultivars of *Glycine max* L. formed by two *Rhizobium fredii* strains. *Cytobios* **50**, 181-190.

Shipton W. A. and Burggraaf A. J. P. (1982) A comparison of the requirements for various carbon and nitrogen sources and vitamins in some *Frankia* isolates. *Plant and Soil* **69**, 149-161.

Shoemaker J. S. and Fitch W. M. (1989) Evidence from nuclear sequences that invariable sites should be considered when sequence divergence is calculated. *Molecular Biological Evolution* **6**, 270-289.

Silvester W. B. and Harris S. L. (1989) Nodule structure and nitrogenase activity of *Coriaria arborea* in response to varying pO_2. *Plant and Soil* **118**, 97-109.

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

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

Simonet P., Normand P., Moiroud A. and Lalonde M. (1984) An improved method for lysis of *Frankia* with achromopeptidase allows detection of new plasmids. *Canadian Journal of Microbiology* **30**, 1292-1295.

Simonet P., Thi Le N., Moiroud A. and Bardin R. (1989) Diversity of *Frankia* strains isolated from a single alder stand. *Plant and Soil* **118**, 13-22.

Simonet P., Thi Le N., Teissier du Cros E. and Bardin R. (1988) Identification of *Frankia* strains by direct DNA hybridization of crushed nodules. *Applied and Environmental Microbiology* **54**, 2500-2503.

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

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

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

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

Smolander A. (1990b) *Frankia* in forest soils. PhD thesis, University of Helsinki, Finland.

Sokal R. R. and Michener C. D. (1958) A statistical method for evaluating systematic relationships. *University of Kansas Scientific Bulletin* **28**, 1409-1438.

References

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

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

Stowers M. D. (1987) Collection, isolation , cultivation and maintenance of *Frankia* (Chapter 2), 29-53. In: G. H. Elkan (ed.) *Symbiotic Nitrogen Fixation Technology*, Marcel Dekker, Inc., New York.

Strunk O. and Ludwig W. (1995) ARB – a software environment for sequence data. Department of Microbiology, Technical University of Munich, Munich, Germany (http://www.mikro.biologie.tu-muenchen.de).

Suharjo U. K. J. and Tjepkema J. D. (1995) Occurrence of hemoglobin in the nitrogen-fixing root nodules of *Alnus glutinosa*. *Physiologia Plantarum* **95**, 247-252.

Swensen S. (1996) The evolution of actinorhizal symbioses: Evidence for multiple origins of the symbiotic association. *American Journal of Botany* **83**, 1503-1512.

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

Swofford D. L. (1991) PAUP: Phylogenetic Analysis Using Parsimony, *User Manual* Version **3.0**s.

Swofford D. L., Olsen G. J., Waddell P. J. and Hillis D. M. (1996) Phylogenetic Inference (Chapter 11), 407-514. In: D. M. Hillis, C. Moritz and B. K. Mable (ed.) *Molecular Systematics*, Sinauer Associates Inc., Sunderland, Massachusetts.

Tajima F. and Nei M. (1984) Estimation of evolutionary distance between nucleotide sequences. *Molecular Biological Evolution* **1**, 269-285.

Tateno Y., Nei M. and Tajima F. (1982) Accuracy of estimated phylogenetic tress from molecular data. *Journal of Molecular Evolution* **18**, 387-404.

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

Thompson J. D., Higgins G. D. and Gibson T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673-4680.

Tjepkema J. (1977) The role of oxygen diffusion from the shoots and nodule roots in nitrogen fixation by root nodules of *Myrica gale*. *Canadian Journal of Botany* **56**, 1365-1371.

Tjepkema J. (1979) Oxygen relations in leguminous and actinorhizal nodules, 175-186. In: J. C. Gordon, C. T. Wheeler and D. A. Perry (ed.) *Symbiotic nitrogen fixation in the management of temperate forests*. Oregon State University Press, Corvallis.

Tjepkema J. D. and Murry M.A. (1989) Respiration and nitrogenase activity in nodules of *Casuarina cunninghamiana* and cultures of *Frankia* sp. HFP020203: Effects of temperature and partial pressure of O_2. *Plant and Soil* **118**, 111-118.

Tjepkema J. D., Ormerod W. and Torrey J. G. (1980) Vesicle formation and acetylene reduction (nitrogenase activity) in *Frankia* sp. CpI1 in culture. *Canadian Journal of Microbiology* **27**, 815-823.

Tjepkema J. D., Ormerod W. and Torrey J. G. (1981) Factors affecting vesicle formation and acetylene reduction (nitrogenase activity) in *Frankia* sp. CpI1. *Canadian Journal of Microbiology* **27**, 815-823.

Torrey J. G. (1978) Nitrogen fixation by actinomycete-nodulated angiosperms. *BioScience* **28**, 586-592.

Torrey J. G. (1987) Endophyte sporulation in root nodules of actinorhizal plants. *Physiologia Plantarum* **70**, 279-288.

Torrey J. G. and Callaham D. (1977) Determinate development of nodule roots in actinomycete-induced root nodules of *Myrica gale*. *Canadian Journal of Botany* **56**, 1357-1364.

Torrey J. G. and Callaham D. (1982) Structural features of the vesicle of *Frankia* sp. CpI1 in culture. *Canadian Journal of Botany* **28**, 749-757.

Torrey J. G. and Racette S. (1989) Specificity among the Casuarinaceace in root nodulation by *Frankia*. *Plant and Soil* **118**, 157-164.

Tremblay F. M. and Lalonde M. (1984) Requirements for in vitro propagation of seven nitrogen-fixing *Alnus* species. *Plant Cell, Tissue and Organ Culture* **3**, 189-199.

Tremblay F. M., Nesme X. and Lalonde M. (1984) Selection and micropropagation of nodulating and non-nodulating clones of *Alnus crispa* (Ait.) Pursh. *Plant and Soil* **78**, 171-180.

Trinick M. J. (1979) Structure of nitrogen-fixing nodules formed by *Rhizobium* on roots of *Parasponia andersonii* Planch. *Canadian Journal of Microbiology* **25**, 565-578.

Troelstra S. R., Lotz L. A. P., Wagenaar R. and Sluimer L. (1990) Temporal and spatial variability in soil nutrient status of a former beach plain. *Plant and Soil* **127**, 1-12.

Uemura S. (1952) Studies on the root nodules of alders (*Alnus* spp.) IV: Experiment on isolation of actinomycetes from alder nodules. *Bulletin of the Government Forest Experiment Station* **52**.

Uemura S. (1971) Non-Leguminous root nodules in Japan. *Plant and Soil* **1**, 349-360.

Ugolini F. C. (1968) Soil development and alder invasion in a recently deglaciated area of Glacier Bay, Alaska, 115-140. In: J. M. Trappe, J. F. Franklin and R. F. Tarrant (ed.) *Biology of Alder*, proceedings of a symposium held at the annual meeting of the Northwest scientific association, Portland.

References

Valla G., Capellano A., Hugueney R. and Moiroud A. (1989) *Penicillium nodositatum* Valla, a new species inducing myconodules on *Alnus* roots. *Plant and Soil* **114**, 142-146.

VandenBosch K. A. and Torrey J. G. (1983) Host-endophyte interactions in effective and ineffective nodules induced by the endophyte of *Myrica gale*. *Canadian Journal of Botany* **61**, 2898-2909.

VandenBosch K. A. and Torrey J. G. (1984) Consequences of sporangial development for nodule function in root nodules of *Comptonia peregrina* and *Myrica gale*. *Plant Physiology* **76**, 556-560.

VandenBosch K. A. and Torrey J .G. (1985) Development of endophytic *Frankia* sporangia in field- and laboratory-grown nodules of *Comptonia peregrina* and *Myrica gale*. *American Journal of Botany* **72**, 99-108.

Van de Peer Y., van de Broeck I., de Rijk P. and de Wachter R. (1994) Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Research* **22**, 3488-3494.

Van der Tang P. (1995) Het kloneren van *Alnus glutinosa* (zwarte els) via weefselkweek, 1-30. Student report of the Netherlands Institute of Ecology, Centre for Terrestrial Ecology, Heteren, The Netherlands.

Van Dijk C. (1978) Spore formation and endophyte diversity in root nodules of *Alnus glutinosa* (L.) Vill. *New Phytologist* **81**, 601-615.

Van Dijk C. (1979) Endophyte distribution in the soil, 84-94. In: J. C. Gordon, C. T. Wheeler and D. A. Perry (ed.) *Symbiotic Nitrogen Fixation in the Management of Temperate Forests*. Oregon State University Press, Corvallis.

Van Dijk C. (1984) Ecological aspects of spore formation in the *Frankia-Alnus* symbiosis. PhD thesis, Leiden State University, The Netherlands.

Van Dijk C. and Merkus E. (1976) A microscopical study of the development of a spore-like stage in the life cycle of the root-nodule endophyte of *Alnus glutinosa* (L.) Gaertn. *New Phytologist* **77**, 73-91.

Van Dijk C., Sluimer A. and Weber A. (1988) Host range differentiation of spore-positive and spore-negative strain types of *Frankia* in stands of *Alnus glutinosa* and *Alnus incana* in Finland. *Physiologia Plantarum* **72**, 349-358.

Van Dijk C. and Sluimer-Stolk A. (1990) An ineffective strain type of *Frankia* in the soil of natural stands of *Alnus glutinosa* (L.) Gaertner. *Plant and Soil* **127**, 107-121.

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

Van Ghelue M. (1994) Interactions in actinorhizal symbioses. PhD thesis, University of Tromsø, Norway.

Van Ghelue M., Lørvaas E., Ringø E. and Solheim B. (1997) Early interactions between *Alnus glutinosa* and *Frankia* strain ArI3. Production and specificity of root hair deformation factor(s). *Physiologia Plantarum* **99**, 579-587.

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

Waterman M. S., Joyce J. and Eggert M. (1991) Computer Alignment of Sequences (Chapter 4), 59-72. In: M. M. Miyamoto and J. Cracraft (ed.) *Phylogenetic Analysis of DNA Sequences*. Oxford University Press, New York.

Watts S. H., Wheeler C. T., Hillman J. R., Berrie A. M. M., Crozier A. and Math V. B. (1983) Abscisic acid in the nodulated root system of *Alnus glutinsosa*. *New Phytologist* **95**, 203-208.

Weber A. (1986) Distribution of spore-positive and spore-negative nodules in stands of *Alnus glutinosa* and *Alnus incana* in Finland. *Plant and Soil* **96**, 205-213.

Weber A. (1990) Host specificity and efficiency of nitrogenase activity of *Frankia* strains from *Alnus incana* and *Alnus glutinosa*. *Symbiosis* **8**, 47-60.

Weber A., Nurmiaho-Lassila E.-L. and Sundman V. (1987) Features of the intrageneric *Alnus-Frankia* specificity. *Physiologia Plantarum* **70**, 289-296.

Weber A., Smolander A., Nurmiaho-Lassila E.-L. and Sundman V. (1988) Isolation and characterization of *Frankia* strains from *Alnus incana* and *Alnus glutinosa* in Finland. *Symbiosis* **6**, 97-116.

Weir B. S. (1990) Phylogeny Construction (Chapter 8), 261-289. In: *Genetic Data Analysis*. Sinauer Associates Inc., Sunderland, Massachusetts.

Wheeler C. T., Crozier A. and Sandberg G. (1984) The biosynthesis of indole-3-acetic acid by *Frankia*. *Plant and Soil* **78**, 99-104.

Wheeler C. T., McLaughlin M. E. and Steele P. (1981) A comparison of symbiotic nitrogen fixation in Scotland in *Alnus glutinosa* and *Alnus rubra*. *Plant and Soil* **61**, 169-188.

Wheeler C. T., Watts S. H. and Hillman J. R. (1983) Changes in carbohydrates and nitrogenous compounds in the root nodules of *Alnus glutinosa* in relation to dormancy. *New Phytologist* **85**, 209-218.

Woese C. R. (1987) Bacterial evolution. *Microbiological Reviews* **51**, 221-271.

Wolters D. J., Akkermans A. D. L. and Van Dijk C. (1997a) Ineffective *Frankia* strains in wet stands of *Alnus glutinosa* L. Gaertn. in the Netherlands. *Soil Biology and Biochemistry* **29**, 1707-1712.

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

Zeng S. and Tjepkema J. D. (1994) The wall of the infected cell may be the major diffusion barrier in nodules of *Myrica gale* L. *Soil Biology and Biochemistry* **26**, 633-639.

Zeng S., Tjepkema J. D. and Berg R. H. (1989) Gas diffusion pathway in nodules of *Casuarina cunninghamiana*. *Plant and Soil* **118**, 119-123.

Zhongze Z., Lopez M. F. and Torrey J. G. (1984) A comparison of cultural characteristics and infectivity of *Frankia* isolates from root nodules of *Casuarina* species. *Plant and Soil* **78**, 79-90.

References

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

Zitzer S. F., Archer S. R. and Boutton T. W. (1996) Spatial variability in the potential for symbiotic N_2 fixation by woody plants in a subtropical savannah ecosystem. *Journal of Applied Ecology* **33**, 1125-1136.

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

Zoon F. C. (1995) Biotic and abiotic soil factors in the succession of sea buckthorn *Hippophaë rhamnoides* in coastal sand dunes. PhD thesis, Wageningen Agricultural University, The Netherlands.

INEFFECTIVE *FRANKIA* IN WET ALDER SOILS

SUMMARY

Although most known *Frankia* are able to fix atmospheric dinitrogen gas, occasionally some strains have been isolated (from root nodules) which are unable to do so, neither free-living, nor in symbiosis with a host plant. Such (Fix^-) *Frankia* strains are called ineffective (in nitrogen fixation), and lack the typical vesicles (both in free-living form and inside nodules), in which nitrogen fixation in effective *Frankia* strains is generally concentrated. Ineffective *Frankia* strains have been isolated from a wide variety of host plant species but until recently, little was known about their ecology, or their phylogenetic position with respect to other *Frankia* strains.

Frankia soil populations can be studied using the nodulation of the root systems of host plants (like *Alnus glutinosa*) as a means of detection (bio-assay). Young alder seedlings are inoculated with a soil suspension, and are harvested after about six to eight weeks, when *Frankia* nodulation has reached its peak. In this way, soil populations of ineffective *Frankia* were first discovered in a wet black alder (*A. glutinosa*) site at Oostvoorne, in the South-western part of the Netherlands, but they could also be encountered at other Dutch wet alder locations (chapter 2a). The distribution of ineffective *Frankia* could not be linked to chemical characteristics of the soil or to the composition of the understory vegetation, and with ineffective *Frankia* present in six out of ten wet alder sites it can be stated that this particular *Frankia* type is a common soil inhabitant of these ecosystems.

A lowering of the soil water-table at the Oostvoorne site was mirrored by the decline of the soil population of ineffective *Frankia*, which provides additional evidence that ineffective *Frankia* in soil are limited to water-saturated soil conditions (chapter 2b).

While nodulation of host plants by *Frankia* is normally a statistically straightforward process, which can be adequately modelled by a Poisson distribution, the nodulation of alder seedlings by ineffective *Frankia* is complicated by the occurrence of resistance of some seedlings to ineffective *Frankia* nodulation. Even a single seed-batch, derived from one alder

tree can be divided into seedlings which are resistant or susceptible to nodulation by ineffective *Frankia*. Apart from the water-saturation of the soil, it is not unthinkable that the ineffective *Frankia* population can also be influenced by the fraction of resistant plants within the alder vegetation.

To determine whether the resistance to ineffective *Frankia* nodulation within natural alder vegetations influences ineffective *Frankia* soil populations, three wet alder sites with, and two sites without an ineffective *Frankia* population were studied using the bio-assay technique mentioned above (chapter 3b). From each location the percentage of resistant seedlings within each of four different seed-lots from individual alder trees was determined, and examined statistically. In case the resistance of the host plants determines the size of the ineffective soil population, the sites with a large ineffective *Frankia* population would be expected to hold the lowest ratio of resistant / susceptible alder seedlings. If, on the other hand, ineffective *Frankia* affect its host plant negatively, an increased percentage of resistant seedlings would be expected at the locations possessing an ineffective *Frankia* soil population.

The research was complicated by the emergence of non-*Frankia* root nodules on the root systems of the test plants, closely resembling ineffective *Frankia* nodules. These 'myco-nodules' were the result of the alder plants being infected by the fungus *Penicillium nodositatum*. Although *Frankia* and the *Penicillium* strain compete for the same zones of infection, it could be shown that the (ineffective) *Frankia* negatively affected *Penicillium* nodulation and not vice versa. From the resistance data it appeared, that the sites with ineffective *Frankia* produced alder seedlots with a higher percentage of resistant seedlings than the sites without an ineffective *Frankia* population.

The pattern of resistance among full-grown alder trees at several of the test locations was studied by using clonally produced alder plantlets. Obtaining clones from mature alder trees proved to be difficult, but a two-step cloning method (chapter 3a) provided sufficient material to confirm some of the findings of the seed-lot resistance experiments (chapter 3b).

Whereas ineffective *Frankia* strains had been isolated from (effective) *Frankia* nodules on several occasions, and also an ineffective *Frankia* strain had been isolated from the ineffective

Frankia nodules derived from soil from the Oostvoorne location, we were unable to isolate ineffective *Frankia* strains from the root nodules that were formed during the experiments of chapter 2a. It is thought, however, that this is more the result of not (yet) knowing the correct isolation conditions, rather than of the inability of ineffective *Frankia* to grow in a free-living mode.

In order to gain an insight into the phylogenetic relationship between ineffective *Frankia*, and the relationship with effective *Frankia* strains, the nucleotide sequence of the 16S rDNA gene of several *Frankia* strains was determined, and a phylogenetic tree was constructed (chapter 4). In chapter 4a some of the theory behind phylogenetic tree-construction is discussed, and a number of computer software packages for the construction of phylogenetic trees are briefly described. Since the ineffective *Frankia* strains under study were not obtained in pure culture, the necessary DNA was isolated directly from ineffective root nodules, amplified using universal 16S rDNA probes, cloned into an *E. coli* vector, and sequenced. Surprisingly, the 16S rDNA from the uncultured ineffective *Frankia* strains not only differed from the effective *Frankia* sequences from the same geographical regions, but were also found to be different from the various ineffective *Frankia* isolates.

In conclusion, the group (uncultured) ineffective *Frankia* strains has been shown to be widely distributed in wet *Alnus glutinosa* vegetations, and is likely to be dependent on water-saturated soils for maintenance of its population size. Based on analysis of resistance patterns of alder seed-lots and clones from mature trees, it was concluded that ineffective *Frankia* are not dependent on the nodulation of *Alnus* for survival, but rather that alder populations could be selected for higher resistance in the presence of an ineffective *Frankia* soil population. Finally, based on 16S rDNA sequences, uncultured ineffective *Frankia* strains have been found to form a (new) monophyletic group within *Frankia*, apart from ineffective *Frankia* isolates.

INEFFECTIEVE *FRANKIA* IN NATTE ELZE-BODEMS

SAMENVATTING

Om hun stikstof behoefte aan te kunnen vullen, zijn sommige planten een symbiotische relatie aangegaan met bacteriën die het vermogen hebben atmosferisch stikstofgas te binden. Deze bacteriën, zoals de vertegenwoordigers van het bekende *Rhizobium* geslacht, kunnen leven in gespecialiseerde organen, wortelknollen genaamd, die deel uitmaken van het wortelstelsel van de plant. Terwijl *Rhizobium* soorten doorgaans geassocieerd zijn met Vlinderbloemigen onderhouden andere, voornamelijk houtige gewassen, symbiotische relaties met bacteriën behorend tot het actinomyceten-geslacht *Frankia*. Omdat veel van deze planten een economische waarde vertegenwoordigen (hetzij direct, hetzij vanwege hun bodemverrijkende eigenschappen) wordt de *Frankia* / waardplant interactie intensief bestudeerd.

Hoewel de meeste bekende *Frankia's* in staat zijn atmosferisch stikstofgas te binden, zijn er af en toe (uit wortelknollen) *Frankia*-stammen geïsoleerd die hiertoe niet in staat bleken, noch in vrijlevende vorm, noch in symbiose met een waardplant. Dergelijke (Fix^-) *Frankia*-stammen noemen we ineffectief (qua stikstofbinding), maar veel meer was niet over hen bekend.

Frankia bodempopulaties kunnen worden bestudeerd door de knolvorming aan de wortelstelsels van waardplanten (zoals *Alnus glutinosa*) te gebruiken als een detectiesysteem (biotoets). Jonge elzenzaailingen worden geïnoculeerd met een grondsuspensie en na zes tot acht weken geoogst, wanneer de knolvorming door *Frankia* op zijn hoogtepunt is. Op deze manier werden bodempopulaties van ineffectieve *Frankia*'s voor het eerst gevonden in een natte zwarte elzenbegroeiing (*Alnus glutinosa*) op Oostvoorne, in het zuidwesten van Nederland. Bij een vervolgonderzoek werd op zes (op een totaal van tien) andere locaties in Nederland eveneens een ineffectieve *Frankia* populatie in de bodem aangetroffen. (hoofdstuk 2a). De verspreiding van de ineffectieve *Frankia*'s was niet direct gekoppeld aan bepaalde chemische bodemkarakteristieken of aan de vegetatiesamenstelling van de onderbegroeiing.

Een daling van het grondwater niveau in de Oostvoorne locatie ging samen met de afname van de bodempopulatie van ineffectieve *Frankia*, hetgeen additioneel bewijs levert voor de hypothese dat het voorkomen van ineffectieve *Frankia* in de grond gelimiteerd is tot waterverzadigde bodemcondities (hoofdstuk 2b).

Terwijl knolvorming bij waardplanten door *Frankia* gewoonlijk een kansproces is, dat goed kan worden beschreven middels een Poisson verdeling, wordt de nodulatie van elzenzaailingen door ineffectieve *Frankia*'s gecompliceerd door het voorkomen van resistentie van sommige zaailingen tegen knolvorming door ineffectieve *Frankia*'s. Zelfs een enkele zaadgroep, afkomstig van één en dezelfde els kan worden verdeeld in zaailingen die resistent of gevoelig zijn voor knolvorming door ineffectieve *Frankia*'s. Behalve de mate van waterverzadiging van de bodem zou ook de verhouding tussen gevoelige en resistente planten in een elzenvegetatie invloed kunnen uitoefenen op de ineffectieve *Frankia* populatie.

Om te bepalen of de resistentie tegen knolvorming door ineffectieve *Frankia* binnen natuurlijke elzenvegetaties invloed heeft op de ineffectieve *Frankia* bodempopulaties, werden drie natte elzengebieden met -, en twee zonder een ineffectieve *Frankia* populatie bestudeerd, waarbij gebruik gemaakt werd van de hierboven genoemde biotoets (hoofdstuk 3b).

Van elke locatie werd het percentage resistente zaailingen van vier verschillende zaadgroepen (elk van een andere elzenboom) bepaald, en vervolgens werden de gegevens statistisch geanalyseerd. Wanneer resistentie van de waardplant de grootte van de bodempopulatie van ineffectieve *Frankia*'s zou bepalen, dan zouden de locaties met een grote ineffectieve *Frankia* populatie de laagste resistente / gevoelige zaailing verhouding moeten hebben. Wanneer, aan de andere kant, ineffectieve *Frankia*'s hun waardplant negatief zouden beïnvloedden dan zou men voor deze locaties een verhoogd percentage resistente zaailingen verwachten.

Het onderzoek werd gecompliceerd door de vorming van wortelknollen die, hoewel ze leken op ineffectieve *Frankia* knollen, veroorzaakt werden door de schimmel *Penicillium nodositatum*. Hoewel *Frankia* en de *Penicillium*-stam elkaar beconcurreren voor dezelfde infectieplaatsen, kon worden aangetoond dat de (ineffectieve) *Frankia* de knolvorming van *Penicillium* negatief beïnvloedde maar dat het omgekeerd niet het geval was. Uit de

resistentiedata bleek, dat de locaties met ineffectieve *Frankia*'s elzenzaad voortbrachten met een hoger percentage resistente zaailingen dan de locaties zonder een ineffectieve *Frankia* populatie.

Het voorkomen van resistentie bij volwassen elzenbomen in een aantal locaties werd bestudeerd door middel van gekloneerde elzenplantjes. Het verkrijgen van klonen van elzen uit het veld was een moeizaam proces, maar een twee-staps kloneringmethode (hoofdstuk 3a) leverde voldoende materiaal op om enkele van de conclusies van de zaadresistentie experimenten te kunnen ondersteunen (hoofdstuk 3b).

Terwijl ineffectieve *Frankia* stammen verscheidene malen geïsoleerd zijn uit (effectieve) *Frankia* knollen, en ook een ineffectieve *Frankia* stam geïsoleerd is uit de ineffectieve wortelknollen die verkregen werden uit experimenten met grondmonsters van locatie Oostvoorne, waren wij niet in staat ineffectieve *Frankia*'s te isoleren uit de wortelknollen die gevormd werden tijdens de experimenten van hoofdstuk 2a. Dit is waarschijnlijk meer het gevolg van onbekendheid met de voor ineffectieve *Frankia*'s vereiste isolatiecondities dan van het onvermogen van ineffectieve *Frankia*'s om te leven buiten de waardplant.
Om een beeld te krijgen van de verwantschap van ineffectieve *Frankia* stammen onderling, en met effectieve *Frankia*'s werden de nucleotide sequenties van het 16S rRNA van verschillende *Frankia* stammen bepaald en onderling vergeleken in een fylogenetische boom (hoofdstuk 4). Hoofdstuk 4a geeft een overzicht van de theorie van het construeren van een fylogenetische boom, en beschrijft kort een aantal fylogenetische computerprogramma's. Omdat van de bestudeerde ineffectieve *Frankia* stammen geen reincultures konden worden verkregen, werd het benodigde DNA rechtstreeks uit de ineffectieve wortelknollen geïsoleerd. Het16S rDNA werd via de PCR techniek vermenigvuldigd met gebruik van universele 16S rDNA probes, in een *E. coli* vector gekloneerd, en gesequenced (hoofdstuk 4b). Verrassend was dat de 16S rDNA sequenties van de niet-geïsoleerde ineffectieve *Frankia* stammen niet alleen verschilden van de effectieve *Frankia* sequenties, afkomstig van dezelfde geografische locaties, maar ook significant verschilden van de 16S rDNA sequenties van de reeds bekende ineffectieve *Frankia* isolaten.

Samenvattend kan geconcludeerd worden dat de (niet-geïsoleerde) ineffectieve *Frankia* stammen een algemeen bestanddeel vormen van de microbiële flora onder natte *Alnus glutinosa* vegetaties, en waarschijnlijk afhankelijk zijn van waterverzadigde bodems voor het handhaven van hun populatiegrootte. Op basis van de analyse van resistentie-patronen van zaadgroepen en klonen van volwassen elzen, werd geconcludeerd dat deze ineffectieve *Frankia* stammen niet afhankelijk zijn van de nodulatie van *Alnus* om te overleven, maar dat in plaats daarvan er selectie op resistentie kan optreden binnen elzenpopulaties in de aanwezigheid van een ineffectieve *Frankia* populatie. De niet-geïsoleerde *Frankia* stammen kunnen op basis van 16S rDNA sequentieanalyse geplaatst worden in een nieuwe monofyletische groep binnen *Frankia*, apart van ineffectieve *Frankia* isolaten.

STELLINGEN
behorende bij het proefschrift

‘Ineffective *Frankia* in wet alder soils’

van
D. J. Wolters

1) Hoewel de symbiose tussen *Frankia* en *Alnus glutinosa* geen ideaal modelsysteem voor (microbieel) fysiologisch onderzoek is, is het beter geschikt voor oecologisch onderzoek dan bijvoorbeeld de symbiose tussen *Rhizobium* en *Pisum sativum*.

2) Polyfenol-vorming is een relevant verdedigingsmechanisme bij de els om wortelpathogenen zoals ineffectieve *Frankia* te weren (Guan *et al.* 1996).

3) If it looks like a duck, and quacks like a duck, it usually is a duck.
If it looks like a *Frankia*, and is isolated from a *Frankia* nodule, it doesn’t necesssarily have to be a *Frankia* (Ramírez Saad *et al.* 1998).

4) Het feit dat ineffectieve *Frankia* stammen niet in één, maar in tenminste twee monophyletische groepen opgedeeld kunnen worden geeft eens temeer aan dat het onverstandig is zonder meer genetische verwantschappen aan bepaalde fenotypische eigenschappen te verbinden (dit proefschrift).

5) Niet alle micro-organismen ontlenen hun bestaansrecht aan een herkenbare functie in het ecosysteem (dit proefschrift).

6) De snelle vooruitgang in het onderzoeksveld van de microbiële oecologie is met name te danken aan de ontwikkelingen op het gebied van moleculair biologische en genetische technieken en denkwijzen.

7) De groter wordende afhankelijkheid van biologisch onderzoek van externe financiering door onder meer Europese instanties heeft een gebrek aan continuïteit en consistentie qua onderzoeksobjecten tot gevolg, en maakt lange termijnplanning een onzekere factor.

8) Wanneer men uitgaat van één gezamenlijke oorsprong van al het leven moet men wel bedenken dat hedendaagse, ‘primitieve’ organismen evenveel eeuwen aan evolutie achter zich hebben als meer ‘ontwikkelde’ organismen.

9) De historische ‘Nederlandse verdraagzaamheid’ waarop men zich soms graag wil beroemen is niet gebaseerd op een verheven filosofie, maar veel meer op pragmatische, economische gronden. Het is dan ook niet waarschijnlijk dat deze verdraagzaamheid een langdurige recessie zal overleven.

10) De Elfstedenvereniging gaat bijna aan haar eigen succes ten onder. Wanneer men elk jaar een elfstedentocht wil houden, zou men kunnen overwegen langs de te volgen route de wegen met ZOAB te bekleden.

RIJKSUNIVERSITEIT GRONINGEN
18 december 1998