

Effect of Inoculation and Leaf Litter Amendment on Establishment of Nodule-Forming *Frankia* Populations in Soil

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High-N₂-fixing activities of *Frankia* populations in root nodules on *Alnus glutinosa* improve growth performance of the host plant. Therefore, the establishment of active, nodule-forming populations of *Frankia* in soil is desirable. In this study, we inoculated *Frankia* strains of *Alnus* host infection groups I, IIIa, and IV into soil already harboring indigenous populations of infection groups (IIIa, IIIb, and IV). Then we amended parts of the inoculated soil with leaf litter of *A. glutinosa* and kept these parts of soil without host plants for several weeks until they were spiked with [¹⁵N]NO₃ and planted with seedlings of *A. glutinosa*. After 4 months of growth, we analyzed plants for growth performance, nodule formation, specific *Frankia* populations in root nodules, and N₂ fixation rates. The results revealed that introduced *Frankia* strains incubated in soil for several weeks in the absence of plants remained infective and competitive for nodulation with the indigenous *Frankia* populations of the soil. Inoculation into and incubation in soil without host plants generally supported subsequent plant growth performance and increased the percentage of nitrogen acquired by the host plants through N₂ fixation from 33% on noninoculated, nonamended soils to 78% on inoculated, amended soils. Introduced *Frankia* strains representing *Alnus* host infection groups IIIa and IV competed with indigenous *Frankia* populations, whereas frankiae of group I were not found in any nodules. When grown in noninoculated, nonamended soil, *A. glutinosa* plants harbored *Frankia* populations of only group IIIa in root nodules. This group was reduced to 32% ± 23% (standard deviation) of the *Frankia* nodule populations when plants were grown in inoculated, nonamended soil. Under these conditions, the introduced *Frankia* strain of group IV was established in 51% ± 20% of the nodules. Leaf litter amendment during the initial incubation in soil without plants promoted nodulation by frankiae of group IV in both inoculated and noninoculated treatments. Grown in inoculated, amended soils, plants had significantly lower numbers of nodules infected by group IIIa (8% ± 6%) than by group IV (81% ± 11%). On plants grown in noninoculated, amended soil, the original *Frankia* root nodule population represented by group IIIa of the noninoculated, nonamended soil was entirely exchanged by a *Frankia* population belonging to group IV. The quantification of N₂ fixation rates by ¹⁵N dilution revealed that both the indigenous and the inoculated *Frankia* populations of group IV had a higher specific N₂-fixing capacity than populations belonging to group IIIa under the conditions applied. These results show that through inoculation or leaf litter amendment, *Frankia* populations with high specific N₂-fixing capacities can be established in soils. These populations remain infective on their host plants, successfully compete for nodule formation with other indigenous or inoculated *Frankia* populations, and thereby increase plant growth performance.

Alders form root nodules in symbiosis with actinomycetes of the genus *Frankia* that have the ability to fix N₂. Since between 70 and 90% of the total nitrogen assimilated by the host plant can be provided by *Frankia* in root nodules, the plant is to a large extent independent of soil nitrogen (11, 23, 28). Alders therefore represent successful pioneer plants frequently coming up after flooding, fires, landslides, glacial activity, and volcanic eruptions (8). They grow on soils with a wide range of

properties (8, 10). They physically enhance the stability of these soils with their well-developed root system (26) and increase nitrogen mineralization rates in soil, thereby enhancing nitrogen availability and thus improving the quality of impoverished soils. Economically, alders are therefore useful for reforestation and reclamation of nitrogen-depleted, nitrogen-limiting soils. They are also used as nurse trees in mixed plantations with valuable tree species, i.e., by interplanting them with suitable tree crops such as walnut, for production of fuel wood and as a source of timber in monocultures (6, 7, 13, 14).

Mixed plantations of *Alnus* or *Elaeagnus* spp. and valuable tree species are a proven silvicultural practice that exploits the ability of actinorhizal plants to increase soil nitrogen contents for subsequent use as nitrogen resource by the tree crops (6, 7). The efficiency of the symbiosis between *Frankia* and woody plants of the genus *Alnus* is largely determined by environmental factors such as the soil pH (5, 15), the soil matric potential

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(9, 41), and the availability of elements such as nitrogen (27, 49) or phosphorus (40, 51). Other factors, however, also help to determine the genotypes of both partners of this symbiosis (19, 37). An improvement in the symbiosis for economic purposes therefore requires the selection of optimal growth sites but also an optimal combination of plants of interest, e.g., forest ecotypes of *Alnus glutinosa* and superior genotypes of *Frankia* as inocula (19, 22, 50).

Recent studies have shown that inoculation of *Frankia* strains is an appropriate strategy to improve the *Frankia*-*Alnus* symbiosis resulting in increased plant growth performance and nitrogen availability (36, 47, 48). Through inoculation, *Frankia* populations can be established in root nodules under conditions that do not favor vesicle formation in nodules formed by the indigenous *Frankia* population (36). Since nodules are perennial, the positive effect of such inoculations can continue over several years. However, for a long-term effect, the introduced strain not only should compete with the indigenous *Frankia* populations for nodule formation but also should remain active in the nodules and survive in soil. The introduced *Frankia* strains must be able to persist in soil in a physiologically active state since only the physiologically active fraction of the *Frankia* soil population is thought to form root nodules (32).

The physiological status of a specific *Frankia* population in soil might be triggered by environmental factors such as the presence of vegetation that favors saprophytic growth of this population and increases its competitive abilities with respect to root nodule formation (32). Plant bioassays have demonstrated that members of the genus *Frankia* survive and remain infective in soils that are devoid of host plants (2, 24, 32, 42–46). This suggests that *Frankia* strains have the ability to grow in soil. Nutrient resources might be obtained from root exudates since it has been shown that *Frankia* strains are able to colonize and grow on the root surface of different host and nonhost plants without addition of exogenous carbon sources (39, 43). Alternative carbon resources might be obtained from the decomposition of organic material such as leaf litter. Leaves of *Casuarina*, for example, have been found to contain compounds that promote growth of *Casuarina*-infective *Frankia* strains (54). In addition, compounds detected in seeds of *A. rubra* are found to enhance nodulation by frankiae (4).

The aim of our study was to determine whether *Frankia* strains inoculated and incubated in soil amended with leaf litter of *Alnus glutinosa* but without host plants for several weeks remain infective and competitive for nodulation on *A. glutinosa* with the indigenous *Frankia* population. Therefore, after the initial incubation period of soils without plants, soils were planted with seedlings of *A. glutinosa*. After 4 months of growth, plants were analyzed for growth performance, for specific *Frankia* populations in root nodules and for N₂ fixation rates. *Frankia* populations in root nodules were analyzed by in situ hybridization with fluorescent probes targeting specific groups of *Frankia* strains (52, 53) and their N₂ fixation rates were quantified using the ¹⁵N dilution method (12).

MATERIALS AND METHODS

Experimental setup. Surface soil samples (down to a depth of 20 cm) were collected from a sandy loam supporting a natural stand of *A. glutinosa* (located in Ettiswil, Switzerland) (52). This soil is characterized by high NO₃⁻ concen-

trations (10 to 20 mM), a low content of organic material (0.02%), and the presence of *Frankia* subgroups IIIa, IIIb, and IV of the *Alnus* host infection group. At the natural site, however, nodules were formed by only subgroup IIIa (52). Freshly sampled soil was cleared of larger particles, e.g., roots and stones, and then sieved (mesh size, 5 mm).

Half of the soil was inoculated with a mixture of pure cultures of *Frankia* strains AgB1.9, ArI3, and Ag45/Mut15, representing *Alnus* host infection groups I, IIIa, and IV, respectively, each at an estimated density of 10⁷ cells g (fresh weight) of soil⁻¹. *Frankia* strains were grown for 4 weeks in P+N medium (33) containing propionate and NH₄Cl as carbon and nitrogen sources, respectively. Cultures were harvested by centrifugation, washed twice in phosphate-buffered saline (PBS; composed of 0.13 M NaCl, 7 mM Na₂HPO₄, and 3 mM NaH₂PO₄, pH 7.2, in water) (16) and homogenized in PBS by repeated passages through a needle (0.6 mm in diameter) with a sterile syringe (17).

One half of each inoculated and noninoculated soil was mixed with leaves of *A. glutinosa* to a final concentration of 1%. Fresh leaves had been collected directly from the trees at several natural alder stands (located by the River Limmat, Switzerland), dried at 120°C for 3 days, and ground with a mortar and pestle to an average particle diameter of approximately 0.5 mm. The soil samples were incubated in a climate chamber (conditions: 16 h of daylight/8 h of night and 20°C during daylight/16°C during night) without plants.

Six weeks after initiation of the incubation, soil of each of the four treatments was divided into 800-g (fresh weight) portions (*n* = 15 each). Each portion was spiked with 12 mg of ¹⁵N-labeled fertilizer ([¹⁵N]NO₃⁻, 98% ¹⁵N enrichment; Cambridge Isotope Laboratories, Andover, Mass.) and subsequently filled into 800-cm³ pots. The addition of [¹⁵N]NO₃⁻ resulted in a 3.8 atom% excess in ¹⁵N in available soil nitrogen (mathematically determined). Pots were planted with approximately 4-week-old seedlings of *A. glutinosa* (L.) Gaertn. that had been germinated and grown in Perlite supplemented with a modified Heller salt solution (20) containing 0.075 μM NO₃⁻ as nitrogen source at pH 5.4 (18) in a growth chamber with a thermoperiod of 24/18°C and a photoperiod of 16/8 h (day/night, respectively). The pots were adjusted to and maintained at a matric potential of -0.01 MPa (36). Plants were grown in the greenhouse, with a thermoperiod of 28/22°C and a photoperiod of 16/8 h (day/night, respectively) for 4 months (December 14, 1998, to April 14, 1999).

Analysis of soil parameters. Anion concentrations (NO₃⁻, NO₂⁻, SO₄²⁻, PO₄³⁻, and Cl⁻) were determined in pore water of soil samples collected (i) at the beginning of the study, (ii) after 2 weeks of incubation without plants, (iii) at planting time 6 weeks after initiation of the experiment, and (iv) at the end of the plant growth experiment, by ion chromatography (Dionex DX-100 ion chromatograph equipped with an IonPac AS4A-SC column; Dionex, Sunnyvale, Calif.) (21). Pore water was obtained as described by Nickel et al. (36). Micromolar anion concentrations in pore water were correlated to water contents and expressed in micromoles per gram of soil (dry weight).

Analysis of plant parameters. Plant height was measured monthly. At the end of the plant growth experiment, shoots, roots, and nodules of the plants were harvested separately (36). Shoots and roots were dried at 105°C for 24 h for dry-weight determination and stable-isotope analysis. For the stable-isotope analysis, the dried plant material was ground to a fine powder with a steel ball mill (Mixer Mill, Retsch MM2000). A DELTA-S isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany), which was coupled via an interface to an EA-1110 elemental analyzer (Carlo Erba, Rodano, Italy), was used to determine total nitrogen and carbon concentrations and ¹⁵N/¹⁴N and ¹³C/¹²C ratios. The isotopic values of the samples were expressed in the delta notation relative to the international standard for carbon (PeeDee Belemnite limestone):

$$\delta^{13}\text{C} [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 10^3 \quad (1)$$

¹⁵N values were expressed as atom% ¹⁵N excess and were calculated from measured δ ¹⁵N values:

$$\text{atom\% excess}_{\text{sample}} = \{ [^{15}\text{N}/^{14}\text{N}_{\text{sample}} / (1 + ^{15}\text{N}/^{14}\text{N}_{\text{sample}})] \times 100 \} - 0.3663 \quad (2)$$

The ¹⁵N/¹⁴N ratio of soil-derived nitrogen was obtained from atom% ¹⁵N excess values of a "non-N₂-fixing" reference *Alnus* plant. This plant showed nitrogen deficiency symptoms (that is, chlorotic leaves) and had only two small nodules (total nodule weight, 10 mg) with *Frankia* populations that belonged to group IIIa. This plant displayed the highest atom% ¹⁵N excess value in leaves of all plants (3.8 atom% ¹⁵N) evidencing a predominant nitrogen accumulation from soil. In our N₂ fixation model, all fixation abilities were minimum estimates, which were internally consistent in terms of treatment comparisons.

$$\text{N}_2 \text{ fixed/N total} = 1 - (\text{atom\% } ^{15}\text{N excess}_{\text{sample}} / \text{atom\% } ^{15}\text{N excess}_{\text{control}}) \quad (3)$$

Analysis of *Frankia* populations in root nodules. For the analysis of *Frankia* populations in root nodules, all nodules were harvested. Nodule lobes were counted, and fresh weights of nodules were determined (36). Nodules were split into lobes, and *Frankia* populations in the lobes were identified by in situ hybridization using Cy-3-labeled oligonucleotide probes targeting the 16S rRNA of members of the domain *Bacteria* (EUB338) (1) or specific sequences on the 23S rRNA insertion of *Frankia* strains AgB1.9 (probe B1.9), ArI3 (probe 23ArI3), and Ag45/Mut15 [probe 23Mut(II)] (52), representing *Alnus* host infection groups I, IIIa, and IV, respectively. Conditions for hybridization, washing, and analysis were as described by Maunuksela et al. (32) and Nickel et al. (36).

Statistical analysis. All data were expressed as means \pm standard deviations and assessed by multiple pairwise comparisons with Tukey's honestly significant difference test (SYSTAT) or two-way analyses of variance (ANOVA) (*Frankia* inoculation \times leaf litter amendment). Before the two-way ANOVA, normality and homoscedasticity of the data sets were checked, and the data, which were expressed as percentages, e.g., *Frankia* infection groups in lobes, were arcsinus transformed. The significance level was set at $P < 0.05$.

RESULTS

Soil parameters. During the 6-week-incubation period of soils without plants, NO_3^- was the only soil parameter changing significantly. Within 2 weeks, the initially high NO_3^- concentration in pore water [$13 \pm 3 \mu\text{mol g of soil}^{-1}$ (dry weight)] had decreased to a concentration below the detection limit at 0.001 mM in soils amended with leaves. After 6 weeks, the NO_3^- concentration in these soils had increased again to the initial concentration. In nonamended soils, the NO_3^- concentrations remained nearly unchanged, close to the original high value during the whole incubation period. Six weeks after initiation of the experiment when planting began, NO_3^- concentrations in all treatments (i.e., leaf litter amended and non-amended, and also inoculated and noninoculated) were comparably high. NO_2^- was not detectable (detection limit 0.001 mM) in all treatments during initial incubation without plants and at the beginning of the plant growth experiment.

At the end of the plant growth experiment that lasted 4 months, no significant differences between the treatments could be detected regarding water content (average of $16\% \pm 8\%$), pH (approximately 7.1), NO_3^- [$22 \pm 11 \mu\text{mol g of soil}^{-1}$ (dry weight)], NO_2^{2-} [$0.1 \pm 0.2 \mu\text{mol g of soil}^{-1}$ (dry weight)], SO_4^{2-} [$0.1 \pm 0.2 \mu\text{mol g of soil}^{-1}$ (dry weight)], PO_4^{3-} (<0.02 mM), and Cl^- [$0.7 \pm 1.3 \mu\text{mol g of soil}^{-1}$ (dry weight)] in the pore water. However, NO_3^- concentrations in soil from single pots varied widely (0.5 to $75.4 \mu\text{mol}$). At this time, carbon contents in soils of all treatments were not significantly different, but the nonamended and noninoculated soil had a slightly lower carbon content with $4.8\% \pm 0.3\%$ than the others with $5.1\% \pm 0.3\%$. However, nitrogen contents differed significantly between amended and nonamended soils. Soils amended with leaves had a nitrogen content of $0.14\% \pm 0.01\%$ but nonamended soils had only $0.12\% \pm 0.01\%$. The atom% ^{15}N excess values of soils of all treatments were not significantly different (0.3 ± 0.2 to 0.6 ± 0.8).

Plant parameters. Leaves of plants growing on noninoculated, nonamended soil were slightly chlorotic in contrast to the dark green leaves of plants from the remaining treatments. Monthly plant height measurements indicated a faster growth of inoculated plants compared to noninoculated plants (Fig. 1). Similarly, plants on soil amended with leaves grew faster compared to plants on soil without leaf amendment. After 4 months of growth, plants on noninoculated, nonamended soil were the smallest plants measuring an average of 26 ± 7 cm

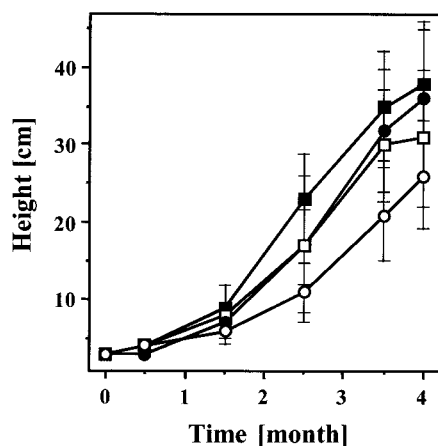


FIG. 1. Averaged plant heights ($X \pm \text{SD}$; $n = 15$ per treatment) of *Alnus glutinosa* cultivated on noninoculated, nonamended soil (○); noninoculated soil amended with 1% *A. glutinosa* leaf litter (●); non-amended soil inoculated with *Frankia* strains AgB1.9, ArI3, and Ag45/Mut15 each at an estimated density of 10^7 cells g of soil $^{-1}$ (dry weight) (□); and inoculated, amended soil (■).

high, while alders on inoculated, amended soil were the largest plants measuring 38 ± 7 cm high. Plants on amended soil were taller than plants on inoculated soil (Table 1). No significant differences were found between plants growing on inoculated and noninoculated soils. However, plants on soils that had been amended and incubated with dried leaves in the soil were significantly taller than plants from soils incubated without leaves (Fig. 1; Table 1). A pattern similar to that of plant height measurements was found for plant dry weights. The lowest dry weights were obtained from plants grown on noninoculated, nonamended soil [2.9 ± 1.3 g of plant material (dry weight)] and the highest ones from inoculated, amended soil plants [5.6 ± 1.6 g of plant material (dry weight)]. Plants from soils either amended with dried leaves or inoculated with pure *Frankia* cultures were 4.3 ± 1.7 g and 4.1 ± 2.5 g (dry weight), respectively. Again, higher values were determined for plants that were grown on soils amended with leaves. The C/N ratios in leaves and roots from the different treatments were determined based on measured C and N concentrations that ranged from 14 ± 1.4 to 16 ± 1.4 to 19 ± 2.3 to 21 ± 3.1 , respectively (data not shown). Plant size was directly correlated with $\delta^{13}\text{C}$ values in plant biomass, since the $\delta^{13}\text{C}$ values of plants growing on noninoculated, nonamended soil were significantly lower with -30.5 ± 0.8 for leaves and -30.1 ± 0.7 for roots. In contrast the values of plants growing on inoculated, amended soil were (-29.2 ± 1.1 and -28.7 ± 1.3 for leaves and roots, respectively) (Table 1).

The atom% ^{15}N excess values of roots were always higher than those of stems and those of stems were always higher than those of leaves (Table 1). The atom% ^{15}N excess values of leaves, roots, and stems from plants grown on inoculated soils were lower than those in plants grown on noninoculated soils. The same was true for plants grown on soils amended with leaves compared to plants grown on nonamended soil (Table 1). The highest values were always detected in plants from noninoculated, nonamended soil and the lowest in plants from inoculated, amended soil (Table 1). The fertilizer uptake of plants, calculated according to equation 2, was not significantly

TABLE 1. Plant parameters^a

Parameter	Nonamended soil (mean ± SD)		Leaf litter-amended soil ^b (mean ± SD)		Effects of leaf litter amendment and inoculation (<i>P</i>) ^c	
	Noninoculated ^d	Inoculated ^e	Noninoculated ^d	Inoculated ^e	Inoculation	Leaf litter
Plant height (cm)	26 ± 7	30 ± 9	36 ± 10	38 ± 7	0.181	<0.001
Plant weight (g [dry wt.])	2.9 ± 1.3	4.1 ± 2.5	4.3 ± 1.7	5.6 ± 1.6	0.028	0.004
C/N ratio of leaves	15 ± 1.4	16 ± 1.3	14 ± 1.4	16 ± 1.4	0.003	0.836
Atom% excess ¹⁵ N values of leaves	1.1 ± 0.6	0.9 ± 0.9	0.7 ± 0.3	0.6 ± 0.5	0.022	0.445
Atom% excess ¹⁵ N values of stems	1.6 ± 0.8	1.2 ± 0.8	1.1 ± 0.5	0.8 ± 0.6	0.178	0.016
Atom% excess ¹⁵ N values of roots	2.0 ± 1.0	1.4 ± 0.8	1.3 ± 0.7	1.3 ± 0.9	0.129	0.018
Plant uptake of soil nitrogen (%)	6.1 ± 2.2	5.9 ± 3.7	6.0 ± 4.0	7.1 ± 4.7	0.494	0.455
Amount of fixed nitrogen in plants (%)	33 ± 19	53 ± 34	58 ± 25	78 ± 24	0.008	0.002
δ ¹³ C values of leaves (‰)	-30.5 ± 0.8	-29.7 ± 1.6	-29.8 ± 0.8	-29.2 ± 1.1	0.075	0.016
δ ¹³ C values of roots (‰)	-30.3 ± 0.7	-29.5 ± 1.8	-29.3 ± 1.2	-28.7 ± 1.3	0.225	0.014

^a The averaged parameters (± standard deviation) were obtained after 4 months of growth.

^b Soil "Ettiswil" mixed with dried alder leaves to a final concentration of 1%.

^c Statistical analysis was performed using two-way ANOVA, and corresponding *P* values are shown.

^d Soil "Ettiswil."

^e Soil "Ettiswil" inoculated with *Frankia* strains ArI3, Ag45/Mut15, and AgB1.9, each at a density of 10⁷ cells g of soil⁻¹ (fresh wt.).

different in all treatments (5.9% ± 3.7% to 7.1% ± 4.7%). Differences, however, were found in the amount of nitrogen in plants originating from N₂-fixation. This amount was only 33% ± 19% when plants were grown on noninoculated, non-amended soil, which was significantly lower than the amount of nitrogen in plants grown on amended only, inoculated only, or inoculated and amended soil, accounting for 53% ± 34%, 58% ± 25%, and 78% ± 24% of the total nitrogen in the plants, respectively (Table 1).

Frankia populations in root nodules. The total weight of nodule lobes per plant was not significantly different in all treatments but the number and size of these lobes varied (Table 2). The number of nodule lobes formed on plants grown on inoculated soil was higher than on plants that had been grown on noninoculated soil. In contrast, the largest nodules were found on noninoculated plants and lobes from plants grown on inoculated soil were significantly smaller. On noninoculated soils, the difference between amended and nonamended soil regarding lobe number and size was not significant (Table 2). However, on inoculated soils a significant difference in the numbers of nodule lobes was found between amended and nonamended soil.

Almost all nodule lobes contained N₂-fixing *Frankia* popu-

lations except for 7 to 17% that did not contain filaments, vesicles, or spores typical for frankiae (Table 1). The numbers of nodule lobes without *Frankia* were not significantly different between the treatments. *Frankia* cells in lobes from plants grown on noninoculated, nonamended soil hybridized with probe 23ArI3 (93% ± 16%) but not with probes 23B1.9 or 23Mut(II) (Table 1). When grown on leaf-litter-amended soil, without inoculation, *Frankia* populations in lobes hybridized with probe 23Mut15 (89% ± 12%) but not with probe 23ArI3 or 23B1.9. In nodules from plants grown on inoculated soils, *Frankia* strains belonging to subgroup IV and IIIa were both identified with probes 23Mut(II) and 23ArI3, respectively. Nodule lobes on plants grown on inoculated, nonamended soil contained high percentages of *Frankia* from subgroups IV (51% ± 20%) and IIIa (32% ± 23%). In contrast, plants grown on inoculated soil amended with leaves had significantly fewer nodules infected by subgroup IIIa (8% ± 6%) than by subgroup IV (81% ± 11%) (Table 2). Frankiae from group I were not found in any nodules.

A significant correlation between N₂-fixing rates and the relative abundance of frankiae was determined for the *Alnus* host infection groups in root nodules (group IV, *R* = 0.52; group IIIa, *R* = -0.57). The linear regression model for the

TABLE 2. Nodulation parameters^a

Parameter	Nonamended soil (mean ± SD)		Leaf litter-amended soil ^b (mean ± SD)		Effects of leaf litter amendment and inoculation (<i>P</i>) ^c	
	Noninoculated ^d	Inoculated ^e	Noninoculated ^d	Inoculated ^e	Inoculation	Leaf-litter
No. of lobes	21 ± 13	31 ± 15	20 ± 13	80 ± 28	<0.001	<0.001
Total lobe weight (mg [fresh wt.])	160 ± 76	149 ± 82	169 ± 68	165 ± 42	0.879	0.652
Average lobe weight (mg [fresh wt.])	9 ± 5	5 ± 1	10 ± 4	2 ± 1	<0.001	0.239
Group IIIa (probe 23ArI3)	93 ± 16	32 ± 23	0	8 ± 6	<0.001	<0.001
Group IV [probe 23Mut15(II)]	0	51 ± 20	89 ± 12	81 ± 11	0.015	<0.001
Group I [probe 23B1.9(II)]	0	0	0	0		
Lobes without frankiae ^e	7 ± 16	17 ± 14	11 ± 12	11 ± 13	0.153	0.957

^a Averaged parameters (± standard deviation) were obtained after 4 months of growth.

^b Soil "Ettiswil" mixed with dried alder leaves to a final concentration of 1%.

^c Statistical analysis was performed using two-way ANOVA, and corresponding *P* values are shown.

^d Soil "Ettiswil."

^e Soil "Ettiswil" inoculated with *Frankia* strains ArI3, Ag45/Mut15 and AgB1.9, each at a density of approximately 10⁷ cells g of soil⁻¹ (fresh wt.).

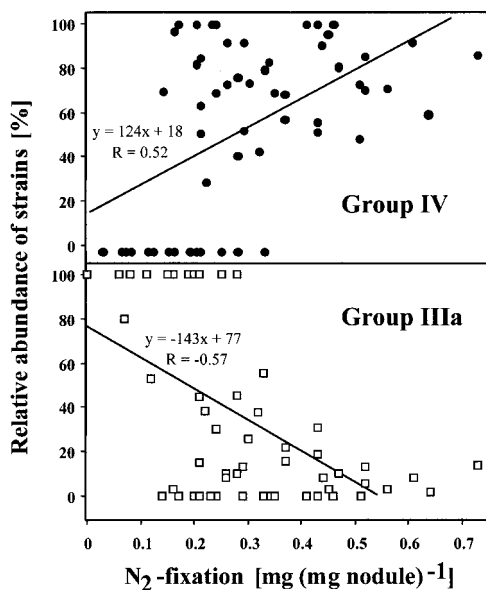


FIG. 2. Correlation between N₂ fixation and relative abundance of frankiae in root nodules of *Alnus* host infection group IV (top [●]) and group IIIa (bottom [□]).

relative abundance of groups IV and IIIa versus N₂ fixation revealed slope coefficients of 2×10^{-3} and -2×10^{-3} and intercept coefficients of 0.18 ± 0.03 and 0.38 ± 0.02 , respectively (Fig. 2). High N₂ fixation was generally associated with those plants characterized by a high abundance of group IV in root nodules, relative to that of group IIIa.

DISCUSSION

The experimental set-up of this study consisted of different pretreatments of the soil before planting of *A. glutinosa* seedlings that included (i) inoculation with pure *Frankia* cultures and (ii) amendment with alder leaf litter. The addition of leaf litter changed soil properties as demonstrated by the large decrease in NO₃⁻ concentrations over time compared to non-amended soils and their subsequent return to the original values. Amending soil with organic material added carbon and nitrogen resources to the soil system supporting the activities of different functional groups of microorganisms. During the initial mineralization of the organic material, prevailing NO₃⁻ was presumably immobilized in microbial cells. The NO₃⁻ concentration might have increased while alder leaves degraded from activity of nitrifying bacteria in the highly oxic soils. Thus, the addition of organic material not only supplied potential carbon and nitrogen sources for *Frankia* populations in soil but also changed additional abiotic and biotic components of the soil.

Plant growth enhancement in this study was less pronounced than in our previous study in which seedlings were planted directly after inoculation with the same strains used in the present study and cultivated with similar water availability (36). The studies also differed with respect to the overall growth conditions because the previous study was conducted under natural light and temperature regimes and this study used controlled light and temperature conditions. Nevertheless, this

study detected the significant effects of leaf litter amendments on plant growth performance, including generally increasing the height and weight of plants. The positive effect of leaf litter amendments on plant growth performance might be due to alterations in physicochemical characteristics of the soils that were not detected in our chemical analyses of the pore waters. During the initial incubation of soil amended with leaf litter, but without plants, mineralization and nitrification processes might have resulted in better availability of nitrogen for seedlings although the NO₃⁻ concentration at the time of planting was comparably high with about $13 \pm 3 \mu\text{mol g of soil}^{-1}$ (dry weight) in all treatments.

In a previous study, we had demonstrated that high NO₃⁻ concentrations in pore water of nonamended, noninoculated soil were not sufficient for optimal plant growth during a 4-month-growth period (36). Leaves of plants grown on these soils were chlorotic and displayed a much higher C/N ratio (25 ± 3) than those of plants grown on nonamended, inoculated soils (16 ± 2) (36). In this study, however, C/N ratios in plant leaves of all treatments were comparably low (14 to 16) based on comparable C and N contents. These values did not indicate any nitrogen limitation but leaves of plants grown on noninoculated, nonamended soil in this study appeared slightly less green than those of plants from the remaining treatments. In contrast to amended soils, NO₃⁻ and NO₂⁻ concentrations in pore water of nonamended soils did not change significantly during the incubation without plants. Therefore, additional factors besides the assumed physicochemical parameters generated after leaf litter amendment and incubation must be assumed to affect nitrogen availability and supply for plants.

Inoculation also had a positive effect on plant growth performance but the effect was less pronounced than in treatments with the leaf litter amendment. It is known that inoculation with *Frankia* strains might improve plant growth performance by enhancing nodule formation on the host plant and by increasing nitrogen availability and supply (17, 36, 47, 48). Plant growth performance in this study correlated to only small differences in nodulation, i.e., the nodule lobe weight showed no significant differences between treatments and the number of nodule lobes obtained was only higher for plants on inoculated, amended soil (Table 2). Total lobe weight of and lobe numbers on plants from nonamended treatments were comparable with those obtained in the previous study (36) except that the total lobe weight of nodules on plants grown on the noninoculated soil was much larger than in the previous study [$160 \pm 76 \text{ mg}$ versus $38 \pm 43 \text{ mg}$ (fresh weight)] (36). Despite only small differences in nodulation, percentages of nitrogen in plants originating from N₂ fixation increased from $33\% \pm 19\%$ when plants were grown on nonamended, noninoculated soil to $78\% \pm 24\%$ when plants were grown on amended, inoculated soil. These results indicate differences in structure and N₂-fixing activity of *Frankia* populations in root nodules of plants grown on soils with leaf litter amendments and with inoculation.

Frankia populations in root nodules on plants grown on noninoculated, nonamended soils represented the population generally found in nodules harvested from plants at the field site (53). This population accounted for only a part of the indigenous *Frankia* population present in this soil that was known to harbor at least three subgroups, IIIa, IIIb, and IV, of

the *Alnus* host infection group (53). In contrast to our previous study in which most of the nodules were found to contain no frankiae (36), nearly all nodule lobes analyzed in this study harbored vesicle-forming frankiae of subgroup IIIa. This could explain the much lower C/N values and greener leaves of plants in our recent study compared to the previous study but may not explain why the percentage of nitrogen in plant leaves originating from N₂ fixation was low with 33% ± 19% of the total nitrogen in the plant.

Leaves and roots from plants grown on amended only, inoculated only, or amended and inoculated soil displayed higher amounts of nitrogen originating from N₂ fixation than those from plants grown on nonamended, noninoculated soil. They were also significantly ¹³C-enriched, displaying more positive δ¹³C values than the plant material grown on nonamended, noninoculated soil. δ¹³C values can provide long-term information on the influence of the nitrogen source on gas exchange characteristics and water balance of the plant since ¹³C-discrimination by plants was found to be negatively correlated with their efficiency of water use (3, 38). The lower ¹³C-discrimination rate of alder plants grown on amended only, inoculated only, or amended and inoculated soil, therefore indicated improved efficiency of water use by plants with high amounts of nitrogen originating from N₂-fixation. This finding was in contrast to the assumption that due to the high respiratory requirements for driving N₂-fixing, the efficiency of water use of nitrogen N₂-fixing plants such as legumes or *Casuarina* is lower than that of plants utilizing mineral nitrogen from soil (25, 31). In these studies, a lower ¹³C-enrichment in N₂-fixing plants than in plants grown on NO₃⁻ and NH₄⁺ indicated a lower efficiency of water use by N₂-fixing plants.

The nodulation capacity of a soil was suggested to be controlled largely by the physiological status of the inhabiting *Frankia* populations, as indicated by infectivity (34, 35). In our study, leaf litter amendment and subsequent incubation for 6 weeks without plants resulted in a large shift of *Frankia* populations in root nodules from subgroup IIIa to subgroup IV and a concomitant increase in N₂ fixation accounting for 58% ± 25% of the total nitrogen in the plant. Since leaves were harvested directly from the tree and subjected to high temperature treatment at 120°C for 3 days before milling and amendment to soils, any potential introduction of additional *Frankia* populations into the soils can be excluded. The large shift in nodule-forming *Frankia* populations after leaf litter amendment and incubation for 6 weeks without plants must therefore be due to changes in the activity of the indigenous *Frankia* populations in soil. Leaf litter amendment and the incubation conditions applied clearly favored growth of a *Frankia* population of group IV of the *Alnus* host infection group over that of frankiae of group IIIa under saprophytic conditions without plants. This was evident for both indigenous as well as introduced populations and correlated with an increase in specific N₂-fixing activity since the amount of nitrogen in plants originating from N₂ fixation increased significantly with higher percentages of nodules harboring frankiae of group IV (Fig. 2).

In nodules of plants grown at field sites, *Frankia* populations identified by in situ hybridization as belonging to group IIIa usually were of the spore (+) type while those of group IV did not form spores (52). Although this correlation might be acci-

dental since only a limited number of sites and nodules were analyzed, they compare favorably with earlier studies in which spore (+) and spore (-) type *Frankia* populations were found to coexist on the same root system (30). In artificial medium, the *Frankia* population of the spore (-) type exhibited larger specific N₂-fixing activity than the population of the spore (+) type. In contrast to our results, however, the introduction of a *Frankia* population of the spore (-) type into soil harboring an indigenous population of the spore (+) type did not result in the development of significant numbers of nodules of the spore (-) type nor in an increase of the N₂ fixation rate. Plant growth of *A. glutinosa* was much more enhanced after inoculation with a *Frankia* strain obtained from nodules of the spore (-) type than after inoculation with homogenates of nodules of the spore (+) type or a mixture of both (29).

In summary, our study has shown that introduced *Frankia* strains incubated in leaf-litter-amended and nonamended soil for several weeks in the absence of plants remained infective and competitive for nodulation with the indigenous *Frankia* populations on the host plant *Alnus glutinosa*. Inoculation into and incubation in soil without host plants generally supported subsequent plant growth performance and increased the percentage of nitrogen acquired by the host plants through N₂-fixation. Further studies, however, need to address long-term effects of such an inoculation on nodulation activity. The potential activation of indigenous *Frankia* populations through increasing the availability of nutrients rather than through inoculation with pure cultures warrants investigation as well.

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