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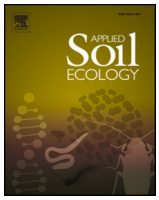
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Soil microbes and nutrient inputs influence root nodulation and tree performance in *Alnus glutinosa*

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ABSTRACT

The symbiotic relationship between the nitrogen-fixing bacteria *Frankia alni* and the pioneer tree species *Alnus glutinosa* plays an important role for tree performance, helping trees thrive in nitrogen-poor environments. However, *F. alni* nodulation can vary greatly between different soils and this could be due to biotic and abiotic characteristics of the soil. Here we examine how microbial communities from young (~10 years) and mature (≥100 years) forests and gradients of available nitrogen (N) and phosphorus (P) influence *F. alni* nodulation and tree performance. In mesocosm experiments, *A. glutinosa* seedlings were inoculated with bacteria, fungi, or both, cultured from young and mature forest soils, alongside *F. alni*. The impacts of N and P availability were examined through controlled nutrient manipulations. Results demonstrated that fungal communities from mature forests suppressed the growth-promoting effects of *F. alni*, although nodule biomass itself was not directly influenced. Further, we isolated and identified bacteria and fungi that were found to contribute to the observed inhibitory effects on *F. alni*-mediated growth promotion. Increased N availability significantly reduced nodule biomass, and we established a threshold at which reliance on symbiosis diminished. Conversely, P-addition stimulated nodulation and tree growth. These findings highlight the pivotal influence of N and P availability in determining the *A. glutinosa-F. alni* symbiosis, while also providing evidence that specific microbes in the soil influence these dynamics.

1. Introduction

Symbiotic relationships between plants and soil microbes, especially nitrogen (N)-fixing bacteria, play a crucial role in plant productivity across terrestrial ecosystems (Carney and Matson, 2005; Van Der Heijden et al., 2008). *Alnus glutinosa* (L.) Gaertn., a pioneer tree species adaptable to nitrogen-poor, early-succession conditions, establishes such a relationship with the ubiquitous actinobacterium *Frankia alni*, enabling it to fix atmospheric nitrogen (Sellstedt and Richau, 2013; Nouioui et al., 2016). Past studies have demonstrated the importance of nitrogen-fixing actinorhizal trees, such as *A. glutinosa* due to their potential to enrich the soil with nitrogen and mitigate biotic stresses, thereby facilitating ecological succession in restoration projects (Schwencke and Carú, 2001; Roy et al., 2007; Diagne et al., 2013). *A. glutinosa* and actinorhizal trees are also used as a pioneer nurse species in forest restoration in places where their high-water demands are met (e.g. northern Europe) and they can rapidly grow on disturbed sites, minimizing the need for nitrogen fertilizers (Diagne et al., 2013; Pedersen et al., 2025). As forests develop after tree planting, both biotic (e.g., bacteria and fungi) and abiotic (e.g., nutrients) characteristics of the soil undergo significant changes (Ritter et al., 2003; Wan et al., 2021). Considering that *A. glutinosa* is a dual mycorrhizal species and associates with a wide range of forest microbes (Boeraeve et al., 2019; Thiem et al., 2023) this can in turn, impact the *F. alni* symbiosis with *A. glutinosa* (Bélanger et al., 2011). Although these changes in biotic and abiotic conditions are relatively well-documented, the impact of changes in soil properties such as microbial community composition, and soil nutrient concentrations on *F. alni* nodulation during forest succession remains understudied.

As forests develop after tree planting, microbial communities in the soil also change and become increasingly more adapted to a forest ecosystem (Gunina et al., 2017; Liu et al., 2020; Guo et al., 2024). Actinorhizal symbionts, which commonly colonize trees, have the potential to interact with other microbes (Godbout and Fortin, 1983;

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Markham, 2005). For example, several past studies highlight that both ecto- (EcM) and arbuscular mycorrhizal (AM) fungi positively impact the development and functioning of the *F. alni* symbioses resulting in increased nodulation (Orfanoudakis et al., 2004, 2010) and enhanced tree performance (Isopi et al., 1994; Oliveira et al., 2005). However, soil microbial communities are much more complex than singular interactions and although studies on how mycorrhiza affect *F. alni* are more well-known, the effects of other members of the microbial community, such as other fungi or bacteria (separately or as part of the whole microbial community) are not. Recent studies have shown that as forests mature, soil communities change, and that *F. alni* nodulation decreases (Georgopoulos et al., 2024) possibly due to the presence of specific microbes in mature forests inhibiting this symbiosis (Georgopoulos et al., 2025). However, our knowledge of how soil microbes affect *F. alni* nodulation is still limited.

Crucially, the availability of soil N and phosphorus (P) themselves is a fundamental indicator of soil nutrient status and potential limitations to tree growth and symbiosis (Gentili and Huss-Danell, 2003; Gong et al., 2020). During forest development, soil available nutrient concentrations shift in tandem with the changing microbial communities, albeit at a slower pace (Gunina et al., 2017; Zhang et al., 2023). Available N and P both follow predictable trajectories during forest development—often declining in late-successional soils as tree uptake outpaces inputs and N and P fertilization inputs are discontinued with conversion from agriculture (Rennie, 1955; Ritter et al., 2003). Typically, N is the primary limiting nutrient for tree growth (Hou et al., 2012). Some trees, like *A. glutinosa*, that associate with N-fixing microbes can obtain N in conditions where available N is scarce but maintaining this symbiosis is also costly for the tree (Chapin et al., 1987; Orfanoudakis et al., 2010). This may lead to trees reducing their investment in their primary obligate symbionts under N-rich conditions and instead taking up available N directly from the soil (Bélanger et al., 2011; Ballhorn et al., 2017). After N, P often is the second most limiting nutrient for tree productivity as plants are often inhibited from N uptake in P deficient soils due to stoichiometric constraints (Israel, 1987). It has also been assumed that P is required for initiating nodulation (Huss-Danell, 1997). Past studies showed that low available P concentrations inhibited *F. alni* nodulation while higher concentrations stimulated nodule biomass production (Ekblad and Huss-Danell, 1995; Yang, 1995; Wall et al., 2000; Gentili and Huss-Danell, 2002), possibly due to P interacting with feedback mechanisms that regulate nodule growth, which is driven by the plant's N demand (Valverde et al., 2002). In one study, adding P even overcame the inhibition from higher concentrations of N, showing that the effects of P were specific on nodulation and not a general stimulation via a plant growth effect (Gentili and Huss-Danell, 2003). This is likely dependent on N:P stoichiometry as reducing N/P ratio has been shown to stimulate nodulation (Wall et al., 2000). While both *F. alni* and *A. glutinosa* are known to persist and nodulate in harsh natural environments, their ability to adapt to gradual shifts of available nutrients remains poorly understood (Bélanger et al., 2011) and the distinct effects of N and P on *F. alni* nodulation are often difficult to disentangle from the soil's biotic characteristics (Georgopoulos et al., 2024). The limited knowledge on the establishment of actinorhizal symbiosis under soil nutrient changes is confounded by the lack of effective methods to monitor the establishment and progression of symbiosis across gradients of nutrient concentrations where most other abiotic and biotic factors are absent. By explicitly framing our experiments in the context of soil N and P gradients, we can better link observed nodulation responses to context dependent responses of *A. glutinosa* to changing nutrient levels.

In this study we investigate the impact of microbial communities, characteristic of young and mature forests, and available N and P concentrations on *F. alni* nodulation associated to the roots of *A. glutinosa*. A mesocosm experiment was established where nodule biomass and tree performance were evaluated by growing trees in the presence or absence of *F. alni,* after inoculating them with bacteria, fungi and bacteria + fungi, cultured from the soils of young (~10 years old) and mature (≥100 years old) forests. Furthermore, microbes were isolated from the roots of some trees to test individual interactions between isolates, *F. alni* and *A. glutinosa*. To investigate the impact of available nutrient concentrations on root nodule density, trees were grown in sterilized soil, across gradients of increasing available N (NH_4NO_3) or P (KH_2PO_4) and in the absence or presence of *F. alni*. We hypothesized that community cultures of bacteria + fungi from mature forests would decrease nodulation and plant performance. We also expected that an increase in available N would lead to a decrease in nodule biomass, while an increase in available P would stimulate nodulation. Finally, we expected that adding high P to high N would potentially stimulate nodule biomass growth through a reduction in the soil N/P ratio compared to fertilizing with just high N.

2. Materials and methods

The aim of this study is to understand the influence of soil biotic (microbial communities, characteristic of young and mature forests) and abiotic (available N and P) characteristics on *F. alni* nodulation and *A. glutinosa* tree performance. Seeds of black alder were obtained from the Dutch nature agency Staatsbosbeheer. The root nodules that were used to make the inoculum for all experiments in this study were collected from a single source: *A. glutinosa* trees that were growing adjacently in natural conditions (small forested area) in Leiden, Netherlands. Nodules were collected fresh each time on the same day that a new experiment was initiated. Only healthy, intact nodules from visibly robust root systems from mature *A. glutinosa* trees were selected for inoculum preparation.

2.1. Microbial community inocula

To obtain microbial inocula, we set up an experiment where communities of bacteria, fungi and bacteria + fungi that originated from forest soils of two distinct developmental stages were cultured in artificial media. These forests were classified based on their planting periods as "young" (2010–2015) or "mature" (1880–1927). Six forests from each developmental stage were sampled in February 2022, resulting in 12 distinct forest sites, each serving as a biological replicate representative of its developmental stage (See SI, Table S1). All forests were located in Drenthe, Netherlands, featured sandy soils, and were initially planted with oak species (*Quercus robur* (L.) and *Quercus petraea* (Matt.) Liebl.). All six of the young forest plantations were planted on agricultural land and shared a similar crop cultivation history. Despite the initial planting with oaks, several other deciduous trees, including *A. glutinosa*, which are often found together with oak (Funk, 1990), were present in all forest stands at the time of sampling. In each forest stand, mineral soil was collected from 10 points at a depth of 15 cm, each point ranging at least 10 m apart. Soil samples from these points were combined to create a homogenized sample for each forest. Additionally, three soil cores (150 cm^3 each, 12 cm deep) were collected from each forest to analyze soil properties using standard methods (see SI, Methods S1; Table S1).

All experiments were conducted in the laboratories of the Institute of Biology at Leiden University. To create community cultures of bacteria, fungi and bacteria + fungi from each forest, we thoroughly mixed phosphate buffer (1 g KH_2PO_4 in 1 l DI H_2O, pH 6.5) with forest soil (1:2, *w*/w) which was then filtered through two decreasing mesh sizes (first 1 mm and then 250 μm) to remove large debris and soil particles. The filtrate from each forest soil was centrifuged (4700 rpm, 10 °C) to form a pellet which was resuspended in LB medium after decanting the supernatant. Each resuspension was stored in a 3:2 ratio of filtrate to glycerol (80 %) and kept at −20 °C (Riis et al., 1998) until the time of preparing the cultures.

To prepare the community cultures, 100 μl of frozen glycerol stocks for each forest was inoculated in tryptic soy broth (TSB) supplemented with the fungicide nystatin (0.1 mg/ml) to create bacterial cultures and

another 100 µl in potato dextrose broth (PDB) supplemented with the antibiotics chloramphenicol (50 mg/ml) and ampicillin (100 mg/ml) to create fungal cultures. Cultures were prepared in duplicate to also create the treatment of bacteria + fungi and were incubated for a week (180 rpm, at 28 °C) in an Innova S44i incubator shaker (Eppendorf, Nijmegen, Netherlands). After one week of incubation, fungal hyphal balls (formed in the fungal cultures) were fragmented and homogenized with sterile tweezers under a laminar flow cabinet. Each culture was centrifuged, and the microbial pellet was resuspended in 50 ml of KH_2PO_4 buffer. For bacteria + fungi-community cultures, 25 ml of bacterial and 25 ml of fungal cultures were combined to standardize microbial volume between treatments. To obtain the initial composition of each community culture, 1 ml of each suspension was centrifuged, and the microbial pellet was stored at −20 °C for DNA analysis. The microbial suspensions were adjusted to 300 ml with KH_2PO_4 buffer and divided into two 150 ml aliquots — one for pots that would later also receive *F. alni* inoculum and one for pots without *F. alni* inoculum. In total, 72 inocula were made (6 young and 6 mature forests × 3 microbial treatments × *F. alni* 2 inoculation conditions). Next, from each of those suspensions, 2 ml was transferred to Eppendorf tubes, where four-week-old *A. glutinosa* seedlings (similar root and shoot length with at least two mature leaves) were left to incubate for 30 min at root level to facilitate seedling inoculation. For seedlings of the control treatment, 2 ml MiliQ H_2O was used instead. These seedlings were previously surface sterilized and germinated in 0.5 MS medium (Kahrizi et al., 2018) to ensure seedlings were not colonized by other microbes (SI, Methods S3).

Following the inoculum, the seedlings were transplanted to 1 l pots (11x11x12cm) filled with gamma-sterilized grassland soil for which soil analysis was also performed as in Georgopoulos et al. (2025) This soil was sandy (98 % sand, 1.4 % silt + clay) with very little gravel (0.6 %), had moderate to low available N levels (NH_4: 37.82 ± 1.61 mg/kg soil; NO_3: 15.41 ± 0.57 mg/kg soil) and low orthophosphate (PO_4: 3.86 ± 0.41 mg/mg soil) as well as slightly basic pH (7.37 ± 0.02) and low SOM (1.85 ± 0.04 %). Immediately after transplantation, the corresponding 150 ml aliquot of the same microbial community culture used for seedling incubation was evenly applied to the soil surface around each seedling. To study the effects of bacteria, fungi and bacteria + fungi communities from each developmental stage on *F. alni* nodulation, half of the pots of each community treatment were additionally inoculated with *F. alni* slurry, which was created fresh on the day of the experimental setup (see: SI, Methods S2). This was done right after inoculating with a community culture, by pipetting 1 ml of nodule homogenate (100 mg nodule tissue / 1 ml H_2O) into a small indentation made right next to the roots of each seedling. Finally, six control pots with gamma sterilized soil received just the phosphate buffer without any inocula while another six controls received the phosphate buffer alongside the *F. alni* inoculum making up the total number of pots to 84.

Plants grew for 12 weeks in a climate room with relative humidity 70 %, light regime of 16 h:8 h (light:dark), air temperature of 20 °C (light) and 18 °C (dark), and were watered three times per week until saturation point of the soil (~30 % of soil volume). During this time, their stem height, number of leaves and chlorophyll were recorded weekly. After 12 weeks, all plants were harvested, and soil subsamples were taken and oven-dried (40 °C) for nutrient analysis. At the harvest, plants were unpotted and their roots were thoroughly rinsed with running tap water. Hereafter, root nodules were counted, cut from the roots with a razorblade and dried at 40 °C to obtain biomass. Additionally, small root subsamples were stored at −20 °C for DNA analysis and larger subsamples were preserved in water at 4 °C, for one day, to be used later for root morphological characterization via scanning. Root scanning was performed using an Epson Perfection V850 Pro scanner at 1200 dpi. Root length, volume and diameter, specific root length (SRL), and the percentage of fine roots (diameter < 0.3 mm) were measured using WinRHIZO software (Regent Instruments, Quebec, QC, Canada). The subsamples were then oven-dried for 96 h (40 °C). Following leaf removal, the stems were separated from the roots, and all components were oven-dried similarly to the root subsamples to obtain aboveground, belowground and nodule biomass. For all experiments in this study, nodule biomass was used instead of the number of nodules or nodule density to avoid redundant metrics (See SI, Fig. S1, Methods S4). Nodule dry weight directly reflects the biomass of nodules, which is closely related to their capacity to fix nitrogen (Aranjuelo et al., 2014). Since nitrogen-fixing nodules contribute more effectively to plant growth as their biomass increases (Fischinger and Schulze, 2010; Fischinger et al., 2010), dry weight offers a more direct measure of this biological function. Both the number of nodules and nodule density may not translate as directly to nitrogen-fixing capacity, as they don't necessarily represent the size or quality of the nodules.

To analyze the N-content of the leaves, a QIAGEN TissueLyser II Bead Mill (Hilden, Germany) was used to grind the oven-dried leaf samples at 370 rpm for 5 min. The percentage of leaf N was measured using the dry combustion method (Matejovic, 1997) with a Thermo Scientific FLASH 2000 CN analyzer (Milan, Italy). For the soil samples from the beginning and the end of the experiment, the soil NH_4^+-N and NO_3^--N concentrations were measured with a spectrophotometer using a standard 1 M KCl extraction method (Kachurina et al., 2000), whereas PO_4^{3-}-P concentrations were determined employing a 0.01 M $CaCl_2$ extraction technique (Houba et al., 2000). The final concentrations were expressed in mg of NH_4^+-N, NO_3^--N, and PO_4^{3-}-P per kg of soil.

2.2. Microbial isolates

Besides evaluating the effect of bacteria, fungal and bacterial + fungal communities on nodulation, we were also interested in the effects of individual microbial species from these communities. Thus, at the time of the harvest, we subsampled small root pieces from the treatments where fungi were added from young and mature forests. We decided to focus on these two treatments after assessing the results of the above-described mesocosms (see results), in an attempt to identify specific microbes that may contribute to the observed patterns. For isolation, roots were ground to a slurry in a mortar using 4 ml MQ H_2O. Then, 50 µl from each root slurry was pipetted and spread onto potato dextrose agar (PDA) plates with bactericide (chloramphenicol), which were incubated in the dark at room temperature (21 °C) for ~10 days. From the resulting growth, morphologically distinct colonies were isolated by streaking them in clean PDA plates until isolates were pure. These were then stored in 80 % glycerol at −80 °C for later inoculation, and also in Cetyltrimethylammonium bromide (CTAB;1:1 ratio) for identification. Although the initial aim was to isolate fungi, some of the isolates were unexpectedly bacteria (see identification below). In total, 14 strains were isolated, of which six (three bacteria and three fungi) were selected from the roots of trees that had received fungal communities from young forests, and eight (two bacteria and six fungi) from those that received fungal communities from mature forests (see, SI, Fig. S2).

To revive the inocula for each strain, 5 µl from the glycerol stocks was added to 2 ml of PDB, and incubated (100 rpm, 21 °C). After one week, 10 µl of each culture was transferred to an Erlenmeyer flask containing PDB to generate biomass. These cultures were then processed as described in the previous experiment. As before, 2 ml of each culture was transferred to Eppendorf tubes (12 tubes per isolate), where four-week-old *A. glutinosa* seedlings of similar root and shoot length and bearing at least two mature leaves—were incubated at the root level for 30 min to facilitate inoculation. Following incubation, the seedlings were transplanted into 1 l pots filled with gamma-sterilized grassland soil. Immediately after planting, 1 ml of the corresponding isolate culture was applied directly to the soil near the seedling roots. Out of 12 seedlings inoculated with each isolate, half were randomly selected to also receive *F. alni* inoculum, while the remaining seedlings did not receive *F. alni* ($n = 6$ replicates). In addition, six pots containing gamma-sterilized soil were set up as controls. The seedlings in these pots did not receive inocula. Another six pots received only the *F. alni* inoculum,

totaling the number of pots to 180. The seedlings were left to grow for 12 weeks, in the same conditions as described before and then harvested as described above.

2.3. Microbial cultures and root-associated microbial communities

Community culture DNA and bacterial isolate DNA were extracted using the DNeasy PowerSoil Pro kit (Qiagen Inc., Hilden, Germany), while DNA from root samples was isolated with the DNeasy Plant Pro kit (Qiagen Inc., Hilden, Germany), following the manufacturer's protocols. For the community cultures, 2 ml of each culture was used for DNA extraction while for the roots, ~100 mg of tissue was used. Fungal isolate DNA was extracted using a CTAB-based method (Schenk et al., 2023). For bacterial analysis, the primers 515F (GTG YCA GCM GCC GCG GTA A) and 926R (GGC CGY CAA TTY MTT TRA GTT T) were used to target the V4 region of the 16S rRNA gene (Quince et al., 2011; Parada et al., 2016). For fungal analysis, the rRNA ITS2 region was amplified using the primers gITS7ngs (GTG ART CAT CRA RTY TTT G) and ITS4ngsUni (CCT SCS CTT ANT DAT ATG C) (Tedersoo and Lindahl, 2016). Prior to sequencing, PCR checks were performed for all reactions. Library preparation was performed at NovoGene UK and sequenced with 2×250 paired-end chemistry in Illumina NovaSeq6000 device. Isolated microbes were sequenced using Sanger sequencing and were identified based on the top five blast results (SI, Table S2). Isolate 6 gave different results for the forward and reverse sequences and was determined visually as *Pseudomonas* sp. by comparing its morphology to other isolated and identified colonies.

2.4. Soil nutrient manipulation

An experiment was established to investigate the hypothesis that increasing nitrogen will inhibit *F. alni* nodulation while increasing phosphorus will increase *F. alni* nodulation. Seedlings were prepared as described above and transferred to 1 l pots inside the climate room under the same conditions and using the same soil as before. A gradient of increasing N was created where pots were fertilized with 7.5, 10, and 20 mM NH_4NO_3 solution (Sigma Aldrich). For the P treatment gradient, pots were fertilized with KH_2PO_4 solution (Sigma Aldrich) at concentrations of 1, 3 and 6 mM. The selected N and P concentrations were designed to span a gradient representative of both nutrient-limited and enriched soils. They were based on, but slightly extended beyond, concentrations used in previous studies investigating the *A. glutinosa–F. alni* symbiosis (Valverde et al., 2002; Ballhorn et al., 2017), in order to identify potential thresholds at which symbiotic investment shifts. These ranges were chosen to simulate realistic ecological conditions, including both natural variation and potential anthropogenic enrichment. For both nutrients, 15 ml of each respective solution was applied to the pots twice a week for a total of 12 weeks (SI, Table S3). Each nutrient concentration was applied to 20 pots. Of these, half of the pots received *F. alni* inoculum, while the remaining pots were not inoculated, serving as fertilized but non-inoculated controls ($n = 10$ replicates). Additionally, 20 unfertilized control pots were prepared, with half the pots receiving only *F. alni* inoculum and the remaining pots left untreated and unfertilized. Lastly, an additional treatment was established using the highest concentrations of NH_4NO_3 (20 mM) and KH_2PO_4 (6 mM) to assess whether phosphorus could mitigate the inhibitory effect of high nitrogen concentrations on *F. alni* nodulation through lowering the N/P ratio. For this treatment, pots were treated similarly, with 15 ml of the high N + P solution applied twice a week for a total of 12 weeks (SI, Table S3). The plants were left to grow for 12 weeks, during which time their stem height, number of leaves and chlorophyll were recorded weekly. After 12 weeks, all plants were harvested and treated similarly to the other experiments.

2.5. Bioinformatics

Sequence processing of 16S (bacteria) and ITS2 (fungi) raw pair-end sequences was performed using PipeCraft2 v1.0.0 (Anslan et al., 2017). The raw data were demultiplexed into sample-specific FASTQ files using the "demultiplexing" module, which employs Cutadapt v4.4 (Martin, 2011). Subsequent steps included quality filtering, denoising, and assembly through DADA2 v1.28 (Callahan et al., 2016) within PipeCraft2. Chimeric sequences were removed using DADA2's consensus-based method, and tag-jumps were corrected with the UNCROSS2 scoring system (Edgar, 2018). For fungi, the ITSx tool (v1.1.3; Bengtsson-Palme et al., 2013) was applied to isolate the ITS2 region, excluding conserved gene fragments. ASVs generated from all libraries were clustered into OTUs at 97 % similarity using vsearch (–iddef = 2; Rognes et al., 2016) and refined further with LULU (Frøslev et al., 2017). Statistical analyses were based on these OTUs, with BLASTn (Camacho et al., 2009) used to generate match lists for post-clustering. Taxonomy assignment varied by library; 16S OTUs were matched to the SILVA v138.1 database (Quast et al., 2012), and ITS2 OTUs were classified using UNITE v9 (Nilsson et al., 2019). Bacterial OTUs classified as chloroplasts or mitochondria and fungal OTUs not assigned to the kingdom Fungi were removed. OTUs with $\geq$80 % identity to phylum-concentration references were classified, though OTUs aligning with Hydrozoa or Porifera at <89 % were similarly categorized as unclassified Metazoa, given their rarity in terrestrial environments.

2.6. Statistical analysis

2.6.1. Community composition of cultures and roots

Community cultures and tree root OTUs were rarefied separately as different extraction kits were used for DNA extraction. OTUs accounting for less than 0.01 % of each samples reads were excluded to enhance the accuracy of community composition analyses before rarefying the datasets (Nikodemova et al., 2023). Bacterial and fungal OTU tables were rarefied to 37,878 and 36,418 reads respectively for cultures and 16,502 and 226 respectively for roots to normalize the sequencing depth. At these sequencing depths, no culture samples were removed from rarefying while three root samples were removed for bacteria and three were removed for fungi. To explore differences in community culture structure between forest development stages, principal coordinate analysis (PCoA) based on Bray-Curtis distances was performed. The analysis used Hellinger-transformed counts and was implemented with the vegdist and pcoa functions from the *vegan* R package (v2.6–4; Oksanen, 2015). PERMANOVA was employed to evaluate variations in bacterial and fungal cultured communities across the two forest developmental stages and four size fractions, also using the *vegan* package. To account for potential biases due to differences in within-group variation, data dispersion was assessed with the betadisper function from the *pairwiseAdonis* R package (v0.4.1; Martinez Arbizu, 2020). When significant differences were detected in the PERMANOVA analysis, pairwise comparisons were performed using the same package. To explore which OTUs were shared between community cultures and the plants they were used to inoculate, Venn diagrams were made using the ps_venn function of the *MicEco* package (v0.9.19).

2.6.2. Effects of community cultures

In the experiment with the microbial community cultures, a linear mixed model (LMM) was used (initially excluding the control) to analyze the interaction between forest developmental stage (young vs mature), microbial community (bacteria, fungi, bacteria + fungi), and *F. alni* inoculation (No *Frankia*, *Frankia*) on each of the measured tree performance variables (see SI, Table S4) using the *lme4* R package v1.1–35.1 (Bates et al., 2015). Forest identity was included as a random factor to account for sampling across various subplots within each forest. A Tukey post-hoc test was used for pairwise comparisons. To examine the effects of inoculating with bacteria, fungi, or both in the presence or absence of

F. alni inoculum, the LMM was rerun, this time including the control as a part of forest development stage treatment (Control, young, and mature). When significant results were found, a Dunnett comparison test in the *DescTools* R package v0.99.51 (Signorell et al., 2023) was used to assess differences compared to the respective control treatments (no *Frankia* vs. no *Frankia* control, and *Frankia* vs. *Frankia* control). The model residuals were evaluated for normality with the Shapiro-Wilk test. A qqplot, and a histogram were used to visually assess data skewness. A Levene test was conducted to check for variance homogeneity across samples. Prior to analysis, square-root transformations were applied to data for aboveground biomass, belowground biomass, nodule weight, and SRL to normalize the data distribution. Pearson correlations were used to examine relationships among nodule biomass, aboveground and belowground biomass, stem width, leaf count, chlorophyll content, percentage leaf N. These analyses were carried out using the *PerformanceAnalytics* R package v2.0.4 (Peterson et al., 2020). Structural Equation Modeling (SEM) was used to test both direct and indirect effects of forest developmental stage (young vs mature) and microbial community (bacteria, fungi, bacteria + fungi) on nodule and aboveground biomass biomass production. SEM was conducted using the *lavaan* package (v0.6–19). Models were estimated via maximum likelihood (ML). We specified direct paths from developmental stage and community to both aboveground biomass and nodule biomass, and an indirect path linking Age and Community → nodule biomass → aboveground biomass. Indirect effects were calculated via the ":=" operator in lavaan. Model fit was evaluated using multiple indices: chi-square test (χ^2), Comparative Fit Index (CFI > 0.95), Tucker–Lewis Index (TLI > 0.95), Root Mean Square Error of Approximation (RMSEA <0.06), and Standardized Root Mean Square Residual (SRMR <0.08). Reported estimates are standardized path coefficients (β), with significance set at $\alpha = 0.05$.

2.6.3. Interactions between microbial isolates and F. alni

Data from the experiment with the microbial isolates were analyzed with a two-way-Anova (initially excluding the control) to analyze the interaction between isolate ID (Iso 2–14) and *F. alni* inoculation (No *Frankia*, *Frankia*) on each of the measured tree performance variables. To examine the effects of inoculation of each isolate in the presence or absence of *F. alni* inoculum, the Anova was rerun, this time including the control as a part of the isolate ID treatment. When significant results were found, a Dunnett comparison test was used to assess differences compared to the respective control treatments (no *Frankia* vs. no *Frankia* control, and *Frankia* vs. *Frankia* control). Lastly, to investigate whether each microbial isolate enhanced or inhibited the aboveground biomass production of *A. glutinosa* when also inoculated with *F. alni*, paired-sample *t*-tests were performed comparing the means of each isolate without and with *F. alni* inoculum using the *dplyr* v2.5.0 R package. The *p*-values were corrected using the False Discovery Rate (FDR) - Benjamini-Hochberg method.

2.6.4. Nitrogen and phosphorus effects

The nitrogen and phosphorus treatments were analyzed separately. However, the treatments share the controls and N + P treatment as they were set up at the exact same time and place and under the exact same conditions. For each, a two-way-Anova was used (initially excluding the control) to analyze the interaction between nutrient treatment (Low, Medium, High, High N + P) and *F. alni* inoculation (No *Frankia*, *Frankia*) on each of the measured tree performance variables. A Tukey post-hoc test was used for pairwise comparisons. Similar to the community cultures experiment. To examine the effects of fertilizing with increasing amounts of nitrogen or phosphorus in the presence or absence of *F. alni* inoculum, the Anovas were rerun, this time including the control as a part of nutrient treatment (Control, Low, Medium, High, High N + P). When significant results were found, a Dunnett's comparison test was used similarly as before. Similarly to before, the *lavaan* package was used in R (ver. 0.6–19) to build SEMs to test the direct and indirect effects of nutrient treatment om aboveground biomass and nodulation. We specified direct paths from nutrient level to both aboveground biomass and nodule biomass, and an indirect path linking Nutrient level → nodule biomass → aboveground biomass.

All graphical visuals were created using the *ggplot2* R package v3.4.4 (Wickham, 2011).

2.7. Accession numbers

The raw Illumina reads are deposited in the National Library of Medicine (NCBI) under accession number PRJNA1272542 (BioSample accessions SAMN48915412- SAMN48915619).

3. Results

3.1. Microbial community composition and richness

Overall, we identified 1344 bacterial and 170 fungal OTUs in the respective community cultures. Among the bacterial OTUs, 395 were shared between young and mature forests, while 660 and 289 were unique to young and mature forests, respectively (SI, Fig. S3A). Similarly, among fungal OTUs, 34 were common to both forest types, with 51 and 85 being unique to young and mature forests, respectively (SI, Fig. S3B).

Among bacterial OTUs detected in tree roots, 72 from the young forest culture were found in trees inoculated with that culture alone, while 64 were present in trees receiving both young forest fungi and bacteria (SI, Fig. S3C). Of these, 55 % belonged to the *Bacillota* phylum, 38 % to *Pseudomonadota*, and 5 % to *Actinomycetota*, with one OTU classified under *Bacteroidota*. In mature forests, 29 bacterial OTUs persisted in roots after inoculation with the corresponding culture, and 31 were detected when both fungal and bacterial inocula were applied (SI, Fig. S3E). These consisted of 63 % *Bacillota*, 24 % *Pseudomonadota*, and 10 % *Actinomycetota*, with one OTU from *Acidobacteriota*. Similarly for fungi, in trees inoculated with the young forest fungal culture, 13 OTUs were found in roots, and this increased to 15 when both fungal and bacterial inocula were introduced (SI, Fig. S3D). All belonged to the *Ascomycota* phylum, with 33 % classified under *Penicillium*, 16 % under *Trichoderma*, and 11 % each under *Arachniotus* and *Fusarium*, while single OTUs were identified from *Talaromyces* and *Humicola*. For the mature forest fungal culture, 15 OTUs were detected in roots following fungal inoculation alone, while 9 remained when both fungi and bacteria were introduced (SI, Fig. S3F). These included 19 % from *Trichoderma* and 12 % each from *Fusarium*, *Umbelopsis*, and *Penicillium*, with individual OTUs assigned to *Sarocladium*, *Humicola*, *Arachniotus*, *Cryptococcus*, *Mucor*, and *Ophiostoma*.

The bacterial (Fig. 1A; PERMANOVA: pseudoF = 6.14, R2 = 0.38, $p = 0.003$), and fungal (Fig. 1B; PERMANOVA: pseudoF = 3.26, R2 = 0.24, $p = 0.012$) community culture composition significantly differed between young and mature forests. Bacterial richness was significantly lower in the cultures from mature forests than the young forests (Fig. 1C), but no differences were found in the fungal richness between the two ages (Fig. 1D). The type of inoculum also significantly affected the community composition of the root associated bacteria (SI, Fig. S4A; PERMANOVA: pseudoF = 1.17, R2 = 0.09, $p = 0.02$) and the root-associated fungi (SI, Fig. S4B; PERMANOVA: pseudoF = 1.42, R2 = 0.11, p = 0.003) of *A. glutinosa* and so did forest age (Bacteria: PERMANOVA: pseudoF = 1.66, R2 = 0.04, p = 0.003; Fungi: PERMANOVA: pseudoF = 2.19, R2 = 0.06, $p = 0.002$). However, pairwise comparisons revealed that while the bacterial and fungal community compositions differed between the two ages and between each inoculated treatment and the control, there were no significant differences among the inoculated treatments themselves, regardless of the community culture used for inoculation. Additionally, the type of inoculum had no effect on root-associated bacterial or fungal richness (SI, Fig. S4C, D).

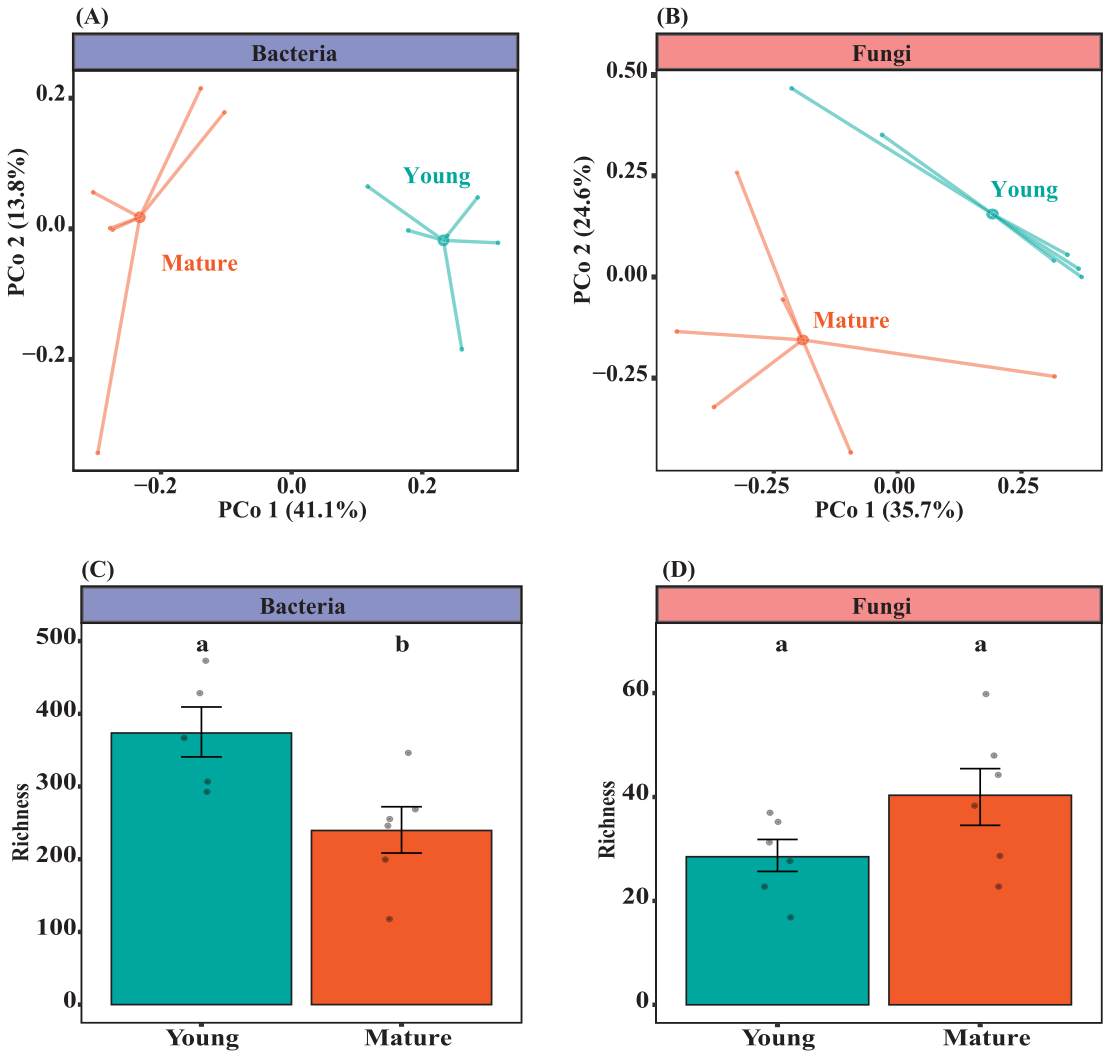


Fig. 1. Spider plots of the composition of the bacterial (A) and fungal (B) community cultures (based on Bray-Curtis similarity) and their mean respective alpha diversity (C, D). In (A) and (B) each point connected with a line represents one forest from the respective age group (Young, Mature). The centroids are also presented. In (C, se = ± 35.03) and (D, se = ± 3.48) each point represents one forest. Letters in the alpha diversity plots indicate significant differences.

3.2. Community cultures experiment

Forest development stage, community culture inoculation and *F. alni* inoculation had a significant interactive effect on *A. glutinosa* root nodule biomass production, aboveground biomass production and leaf N concentration (Fig. 2A, B, C; SI, Table S4). Despite this, a Tukey post-hoc test revealed no significant differences in the nodule biomass production between the different treatments. Although half the trees that were inoculated with each community culture did not receive *F. alni* inoculum, their roots still nodulated (Fig. 2A), suggesting that *F. alni* was present in every culture even though it was not detected in the sequenced DNA. Additionally, trees that received only fungal and only bacterial + fungal cultures (without *F. alni* inoculum) from young forests produced 30 % and 68 % less nodule biomass, respectively, than the ones that received only bacteria, while the addition of *F. alni* inoculum made nodule biomass indistinguishable between treatments but still lower than the control + *F. alni*. Similarly to nodule biomass, trees that were inoculated with bacteria + fungi cultures from young forests without the addition of *F. alni* inoculum exhibited the lowest aboveground biomass production (Fig. 2B). On average, trees that were inoculated with *F. alni* produced 6 % more aboveground biomass than those that were not inoculated. Although the leaf N concentration was indistinguishable between most treatments, trees that were inoculated with fungal cultures from mature forests and with *F. alni* exhibited the highest percentage of leaf N (Fig. 2C). As expected, trees that produced more nodule biomass also produced more above- and belowground biomass and had thicker stems, a higher number of leaves, and higher chlorophyll and leaf N concentrations (SI, Fig. S5). No significant effects of forest development stage, community culture inoculation or *F. alni* inoculation were observed on root/shoot ratio, percentage of fine roots, SRL or soil NH_4^+-N (SI, Table S4). Virtually no NO_3^--N was measured in the soil at the end of the experiment.

When the control treatment was added as a factor in the forest developmental stage and community culture inoculation, the same significant interaction effects were observed on *A. glutinosa* root nodule biomass production, aboveground biomass production and percentage leaf N (Fig. 2A, B, C; SI, Table S5). Specifically, trees that received bacterial cultures from young forests and fungal cultures from mature forests but not *F. alni* inoculum, produced significantly more nodule biomass than the control without *F. alni*. Interestingly, trees that were inoculated with both fungal cultures from mature forests and *F. alni* produced significantly less biomass and had significantly higher leaf N than the control with *F. alni* despite no observed differences in nodule biomass production. Lastly, the trees that received community cultures from either young or mature forests without the addition of *F. alni* had significantly higher leaf N than the control without *F. alni* but were

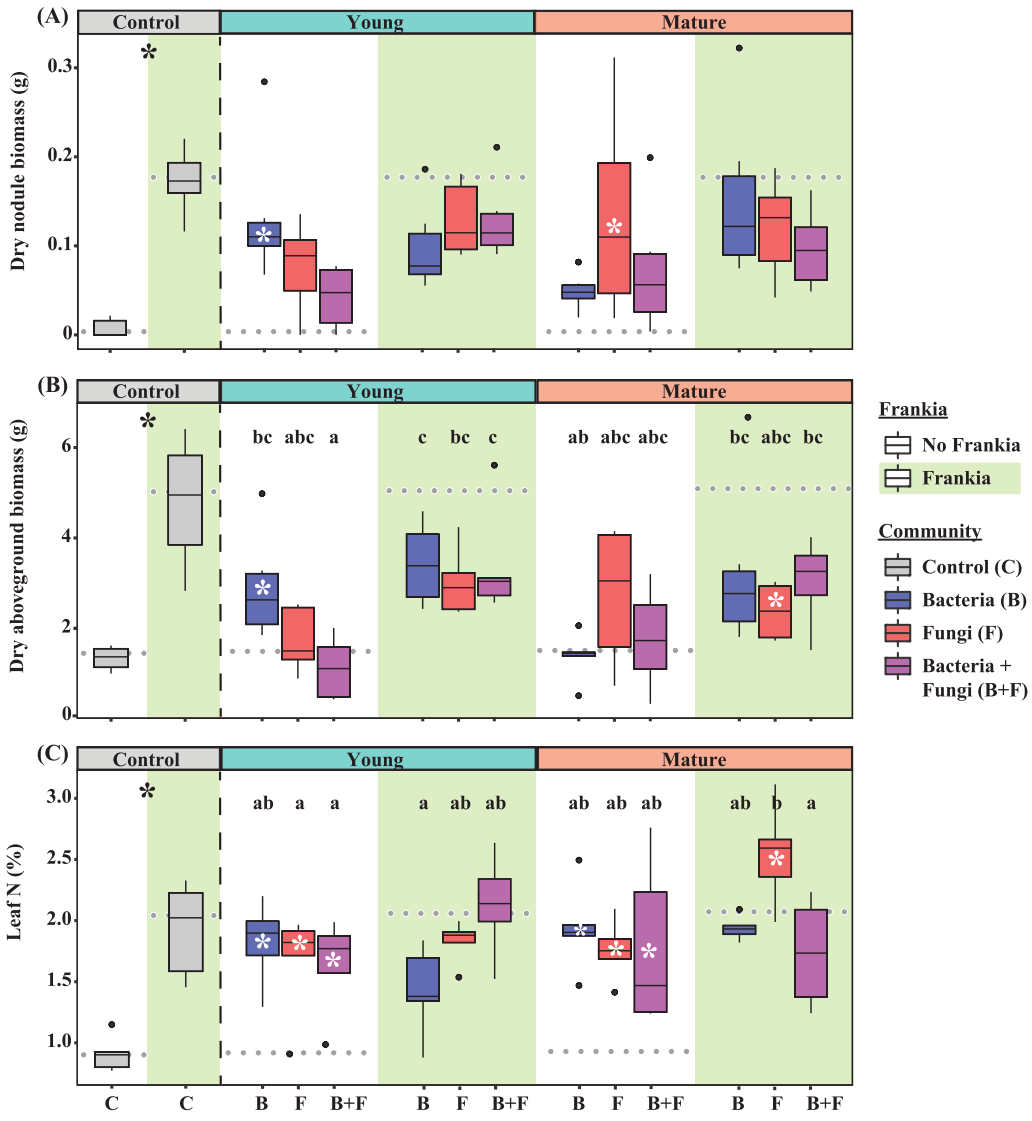


Fig. 2. The effects of forest developmental stage (young, mature), microbial community culture (Bacteria, Fungi, Bacteria + Fungi), *Frankia alni* inoculation (*Frankia*, No *Frankia*), and their interaction on (A) nodule biomass, (B) aboveground biomass, and (C) leaf nitrogen concentration (%) in *A. glutinosa*. Each boxplot displays the median (horizontal line inside the box), interquartile range (25th to 75th percentile), and 95 % confidence interval (whiskers). Grey dotted horizontal lines indicate the mean of each respective control group: controls without *F. alni* are shown in the white background area, and controls with *F. alni* in the green background area. These means were calculated from $n = 6$ replicate plants per control. Statistical comparisons are presented as follows:
Lowercase letters above the boxplots indicate statistically significant differences among all *treatment combinations* (excluding controls), based on Tukey's HSD post-hoc test following a significant three-way interaction in the linear mixed model (LMM).
White asterisks inside boxplots indicate treatments that differ significantly from their respective control group (either with or without *F. alni*), based on a Dunnett's test comparing each treatment to its matching control (e.g., "No *Frankia*" treatments are compared to the "No *Frankia*" control, and likewise for "*Frankia*").
A black asterisk between the two control bars denotes a statistically significant difference between the control groups with and without *F. alni*.

indistinguishable from the *F. alni* control when they were inoculated with *F. alni* (with the exception of trees inoculated with fungi from mature forests).

SEM was used to test whether developmental stage and microbial community composition influenced nodule weight and aboveground biomass, and nodule weight biomass mediated any of these effects. Neither developmental stage ($\beta = -0.059$, $p = 0.612$) nor community composition ($\beta = 0.126$, $p = 0.340$) had statistically significant direct effects on nodule weight. Similarly, no direct effects of developmental stage or community on aboveground biomass were detected. However, nodule biomass had a significant positive effect on aboveground biomass ($\beta = 0.295$, $p = 0.013$. None of the indirect effects of developmental stage ($p = 0.619$) or community ($p = 0.374$) on aboveground biomass via nodule biomass were statistically significant (Table S6).

3.3. Microbial isolates experiment

All the plants that received *T. harzianum* 1 died and were excluded from the analysis. The remaining isolates lead to varying tree mortality (see; SI, Table S7). Isolate identity and *F. alni* inoculation had a significant effect on the above- and belowground biomass production as well as the percentage of fine roots and the SRL of *A. glutinosa* trees (SI, Table S8). In the absence of *F. alni* inoculum, none of the isolates resulted in trees producing more aboveground biomass than the control. From the trees that received *F. alni* inoculum, only those that were inoculated with *T. harzianum* 6, *Luteibacter* and *Pseudomonas* sp. 1 produced significantly more nodule biomass compared to their counterparts that were not inoculated with *F. alni* (Fig. 3A; SI, Table S9). Of these, only *T. harzianum* 6 and *Luteibacter* also produced significantly more

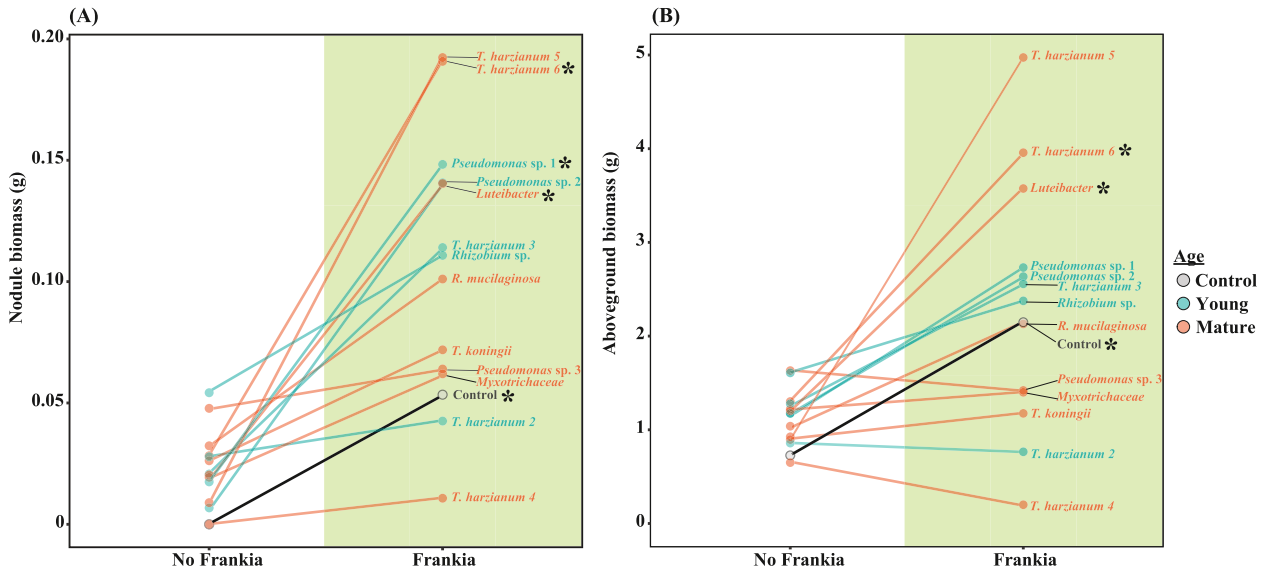


Fig. 3. Comparison of the effect of 13 microbial isolates from young or mature forest soils with and without *Frankia alni* on (A) nodule biomass and (B) aboveground biomass. Each point represents the mean response of six replicate trees, with the x-axis indicating treatment condition ("with *Frankia*" or "no *Frankia*") and the y-axis showing the mean response value for each biomass type. Paired *t*-tests were performed to compare outcomes between *Frankia* and No *Frankia* treatments for each microbial isolate. A black asterisk indicates a significant difference between the *Frankia* and no *Frankia* treatment of each respective isolate or control. The standard error bars of each point have been omitted as to not clutter the visual. No differences were observed between the means of the isolates against their respective control.

aboveground biomass when inoculated in combination with *F. alni* (Fig. 3B; SI, Table S9). Despite this, Dunnett comparisons revealed that none of the treatments produced significantly more nodule or aboveground biomass in comparison to their respective control (No *Frankia* or *Frankia*). Although trees that did not receive *F. alni* inoculum still nodulated, *F. alni* inoculation alone significantly increased root nodule biomass production and leaf chlorophyll concentrations. Specifically, trees inoculated with *F. alni* produced 366 % more nodule biomass and had 46 % more chlorophyll than those that were not inoculated. Trees that produced more nodule biomass also produced more above- and belowground biomass, more leaves, had thicker stems and higher chlorophyll concentrations and a lower root/shoot ratio (SI, Fig. S7). There was no interaction effect of isolate identity and *F. alni* inoculation on any of the measured variables (SI, Table S8). When the control treatment was added as a factor in the isolate identity, the same significant effects as without the controls were observed (SI, Table S10). Despite this, Dunett comparisons revealed no differences between any of the treatments and their respective controls. As expected, trees in the control treatments that were not inoculated with *F. alni* did not nodulate and produced significantly less biomass than the controls with *F. alni* (Fig. 3A, B; SI, Table S9). Although half the trees that were inoculated with each isolate did not receive *F. alni* inoculum, their roots still nodulated (Fig. 3A).

3.4. Nitrogen and phosphorus addition

F. alni inoculation and nitrogen concentration had a significant interaction effect on the nodule biomass production, aboveground biomass production, percentage of leaf N (Fig. 4A, B, C; SI, Table S11) and the SRL of *A. glutinosa* (SI, Table S11). More specifically, nodule biomass production declined with increasing N concentrations when trees were inoculated with *F. alni.* At the highest nitrogen concentrations nodule biomass production was similar to that of trees that were not inoculated (Fig. 4A). As expected, aboveground biomass production and leaf N increased with increasing nitrogen concentrations when trees were not inoculated with *F. alni.* Both were highest when fertilized with the highest N concentration, at which point they reached the same performance as the trees that were inoculated with *F. alni* (Fig. 4B, C). The aboveground biomass production and leaf N of trees that were inoculated with *F. alni* were similar at all concentrations of N fertilization. There were no significant effects of *F. alni* inoculation, nitrogen concentration or their interaction on belowground biomass production, root/shoot ratio, percentage of fine roots or NH_4^+-N in the soil at the end of the experiment (SI, Table S11). No correlations were found between nodule density and above- or belowground biomass production for the nitrogen experiment.

When SEM was used to test the direct and indirect effects of the different N levels, aboveground biomass was significantly increased by the High N ($\beta = 0.351$, $p = 0.006$) and NP ($\beta = 0.267$, $p = 0.033$) treatments. The Medium N treatment had no significant effect ($\beta = -0.048$, $p = 0.707$). Nodule weight was negatively affected by the High N treatment ($\beta = -0.584$, $p < 0.001$) and NP ($\beta = -0.549$, $p < 0.001$) treatments. Nodule biomass had a significant positive effect on aboveground biomass ($\beta = 0.409$, $p < 0.001$). Indirect effects of nitrogen treatment on nodule weight were insignificant (Table S12).

When the control treatment was added as a level in the nitrogen treatment, the same significant interaction effects were observed on *A. glutinosa* root nodule biomass production, aboveground biomass production and leaf N concentration (Fig. 4A, B, C; SI, Table S133). Trees that were inoculated with *F. alni* produced significantly less nodule biomass than the unfertilized control with *F. alni*, already when receiving the medium N concentration. Fertilized treatments without *F. alni* produced more aboveground biomass and had higher leaf N than the unfertilized control without *F. alni* (Fig. 4B, C). Virtually no NO_3^--N was measured in the soil at the end of the experiment.

There was a significant interactive effect for *F. alni* inoculation and the phosphorus treatment on the nodule biomass production, aboveground biomass production, percentage of leaf N (Fig. 4A, B, C; SI, Table S14), as well as chlorophyll concentrations, belowground biomass, root/shoot ratio, percentage of fine roots and the SRL of *A. glutinosa* (SI, Table S14). Nodule biomass and aboveground biomass production were positively correlated ($R = 0.65$, $p < 0.001$) and both increased with increasing phosphorus concentrations when trees were inoculated with *F. alni* (Fig. 4A, B). Trees produced most nodule biomass and aboveground biomass when they were inoculated with *F. alni* and received the highest P concentration and this was significantly higher than the lowest P concentration with *F. alni*. Despite a positive correlation between leaf N and nodule biomass production ($R = 0.63$, $p <$

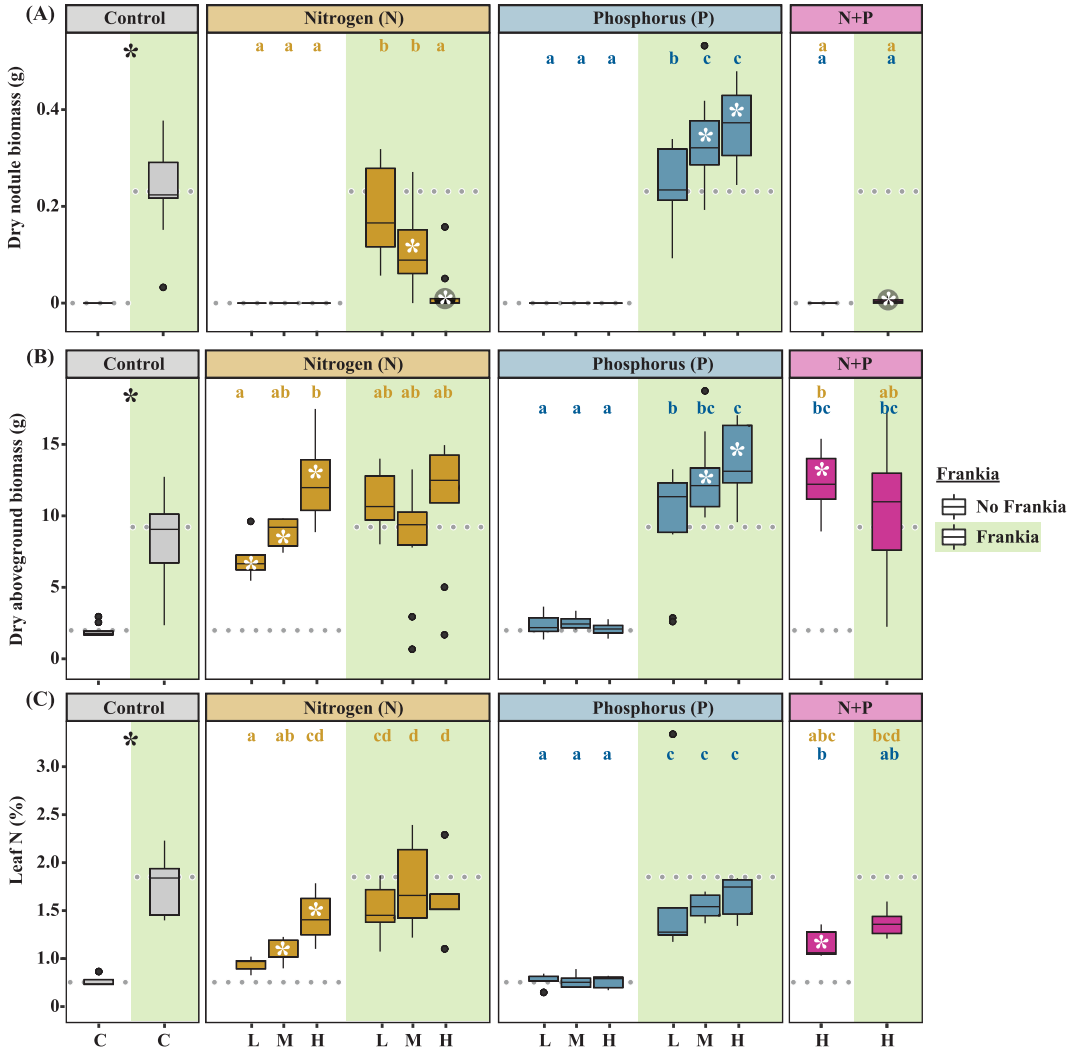


Fig. 4. The effects of nitrogen or phosphorus concentration (Low (L), Medium (M), High (H), and combined High N + P), *Frankia alni* inoculation (*Frankia*, No *Frankia*), and their interaction on (A) nodule biomass, (B) aboveground biomass, and (C) leaf nitrogen concentration (%) in *Alnus glutinosa*.
Each boxplot displays the median (horizontal line), interquartile range (box: 25th–75th percentiles), and 95 % confidence interval (whiskers). Grey dotted horizontal lines indicate the mean of the respective control group: controls without *F. alni* are shown in the white background area; controls with *F. alni* are shown in the green background area. These control means were calculated from $n = 10$ replicates per group. Statistical comparisons are presented as follows:
Lowercase letters above the boxplots denote statistically significant differences among nutrient treatments within each experiment (nitrogen or phosphorus), based on Tukey's HSD post-hoc test following a significant interaction in the linear model (LM). To distinguish between experiments, brown letters represent comparisons in the nitrogen experiment, and blue letters represent comparisons in the phosphorus experiment.
White asterisks inside boxplots indicate a significant difference between a treatment and its respective control group (i.e., either *Frankia* or No *Frankia*), based on Dunnett's test.
A black asterisk between the control bars marks a significant difference between the two controls (with vs. without *F. alni*).
Note: The nitrogen and phosphorus experiments were analyzed separately but share the same control and High N + P treatment groups, as all treatments were conducted simultaneously under identical conditions.

0.001), leaf N was higher in the treatments with *F. alni* than those without *F. alni* but was not significantly different among the different P concentrations. The trees that produced more nodule biomass also produced more leaves and had higher chlorophyll concentrations and thicker stems as well as lower SRL and root/shoot ratios (SI, Fig. S7). The phosphorus treatment had a significant effect on the soil PO_4^{3-}-P at the end of the experiment but post-hoc tests revealed no significant differences between treatments.

When SEM was used to test the direct and indirect effects of the different P levels, aboveground biomass was not directly or indirectly affected by P. The direct effects of Medium ($\beta = 0.131$, $p = 0.320$) and High P ($\beta = 0.157$, $p = 0.234$) levels were small and non-significant (Table S15).

When the control treatment was added as concentration in the phosphorus treatment, the same significant interaction effects were observed on *A. glutinosa* root nodule biomass production, aboveground biomass production and leaf N concentration (Fig. 4A, B, C; SI, Table S16). Trees that were fertilized with medium or high P concentrations and inoculated with *F. alni* produced significantly more nodule and aboveground biomass than the control trees with *F. alni* (Fig. 4A, B). On the other hand, the biomass production of trees that were fertilized with P without *F. alni* inoculation was not significantly different from that of control trees without *F. alni* (Fig. 4A, B).

Finally, the addition of both high N and P resulted in the near absence of nodules when trees were inoculated with *F. alni*, similar to the case for inoculated trees fertilized with high N only (Fig. 4A). Fertilization with both high N and P and no *F. alni* had similar results as fertilizing with just high N. In short, it resulted in significantly higher

aboveground biomass production and leaf N concentration than the control without *F. alni* and the trees that were fertilized with just P (Fig. 4B, C).

4. Discussion

This study examined how shifts in forest soil microbial communities and nutrient availability influence *F. alni* nodulation on the roots of *A. glutinosa* and the subsequent impacts on tree performance. Our findings demonstrate that, while available N and P exert a strong direct influence on nodulation by either inhibiting it (increasing N) or promoting it (increasing P), biotic factors—particularly the soil microbial communities—modulate plant performance more indirectly by suppressing the effects of *F. alni*. Moreover, we provide evidence that isolated fungi and bacteria from forest soils have the ability to suppress the positive effects of *F. alni* on biomass production.

Our results reveal that soil microbial communities derived from young and mature forests do not directly influence nodulation when bacteria and fungi are applied separately and with the addition of *F. alni* inoculum. However, trees that were inoculated with bacteria from young forests or fungi from mature forests, without the addition of *F. alni* inoculum, produced significantly more nodule biomass than the respective, non-inoculated control. This comes as no surprise for the bacteria treatment in which *F. alni* is expected to be present. The presence of *F. alni* in the fungal treatment (where bactericide was added) could be attributed to the inefficiency of the bactericide to completely remove these bacteria. *F. alni* transfer with the *A. glutinosa* seeds is unlikely as nearly none of the trees in the controls without *F. alni* inoculation nodulated. It is possible that *F. alni* to some degree is resistant to chloramphenicol and ampicillin, similar to its resistance to the natural bactericides that *A. glutinosa* exudes (Seidel, 1972). Additionally, as shown from the results of a pilot experiment (SI, Methods S2, Table S17, S18), lower concentrations of *F. alni* seemingly do not affect nodule biomass production, highlighting that as long as a low amount of *F. alni* survives the bactericide treatment, nodulation will still occur (i.e. as observed in all treatments). It is also important to note that two of the mature forest sites had unusually high SOM and moisture content, potentially reflecting the thick organic layers that were observed during their sampling. While such variation reflects natural heterogeneity within mature forest ecosystems, it may have influenced microbial community composition and, consequently, tree responses. When trees were inoculated with *F. alni* alongside the forest microbial communities, no significant differences in nodule biomass production were observed compared to the *F. alni*-inoculated control. However, in partial agreement with our hypothesis and the results of other recent work (Georgopoulos et al., 2025), when bacteria and fungi from mature forests were inoculated together, root nodule biomass production declined compared to when bacteria or fungi were inoculated alone and compared to the *F. alni*-inoculated control. This suggests that either increasing microbial competition (Kuzyakov and Xu, 2013), or more likely, a developmental-stage-dependent difference in microbial community composition (Kang et al., 2018) exerts a negative impact on root nodule formation, albeit not a significant one. Additionally, the latter may be unlikely, as despite inoculating with microbial communities that were compositionally distinct, the root-associated microbial communities that established in *A. glutinosa* were largely similar across treatments. This suggests that *A. glutinosa* is highly selective, shaping the final microbial assemblage regardless of the inoculum source. Alternatively, it could also mean that the low number of taxa that are added by the community culture inocula contribute very little to the overall root community composition compared to microbes that passively colonize the sterilized soils as is apparent from the considerable number of unique OTUs even between treatments that received the same inocula.

Remarkably, despite a lack of significant differences in the nodule biomass production between the different treatments, trees that were inoculated with fungal communities from mature forests and *F. alni* produced significantly lower aboveground biomass than the *F. alni*-inoculated control. Although the biomass and nodule production of trees followed the same pattern in treatment effects and were closely positively correlated, this was not the case for trees that were inoculated with fungal cultures from mature forests. Inoculation with these fungal cultures alone (without *F. alni*) had no significant effect on aboveground biomass compared to the uninoculated control. However, co-inoculation of these cultures with *F. alni* resulted in a significant reduction in aboveground biomass production relative to inoculated *F. alni* alone. This suggests that *F. alni* is primarily responsible for the increased biomass production of *A. glutinosa* and even though biotic factors may not directly affect *F. alni* nodulation, fungi from mature forests have the potential to suppress the growth promoting effects of *F. alni* on *A. glutinosa*.

Interestingly, our results from the follow-up experiment pointed to some fungi from the roots of trees that potentially contributed to the suppressive effects observed from the mature fungal communities. More specifically, *R. mucilaginosa, Myxotrichaceae, T. koningii* and *T. harzianum* 4 and *T. harzianum* 5 suppressed aboveground biomass production when they were co-inoculated with *F. alni*, but had no effects when inoculated without *F. alni.* Although the controls that were co-inoculated with *F. alni* produced significantly more biomass than the non-inoculated controls, this was not observed for the aforementioned fungi where trees appeared to produce the same biomass regardless of *F. alni* co-inoculation. Notably, the nodule biomass produced by these trees did not differ from the control treatment. This suggests that, while these fungi have minimal impact on root nodule biomass or aboveground biomass production when inoculated alone, they somehow suppress the aboveground biomass, probably due to inhibition of the nitrogen transfer. The same was observed for *T. harzianum* 2, *Rhizobium* sp. and *Pseudomonas* sp. from young forests. These observations are surprising for *T. harzianum* and *T. koningii* as both have previously been reported to be highly beneficial, fungal-pathogen-repelling, plant growth promotors (Hadar et al., 1984; Adams et al., 2007; Amira et al., 2017; Promwee et al., 2017; Urbez-Torres et al., 2020), while *Pseudomonas* and *Rhizobium* have been found to inhibit tree growth depending on the species (e.g. *P. syringae* and *R. rhizogenes*; Lamichhane et al., 2014; Gutiérrez-Barranquero et al., 2019; Junta et al., 2021). In contrast to the aforementioned, suppressive isolates, inoculation with *T. harzianum* 6 and *Luteibacter* from mature forests led to more biomass production when co-inoculated with *F. alni* but the biomass production was not higher than the *F. alni*-inoculated control. Although bacteria of the genus *Luteibacter* have been found to promote root development in barley (Guglielmetti et al., 2013), to the best of our knowledge, reports of their effects on trees are lacking. The observed variability in the effects of *T. harzianum* aligns with prior findings suggesting that symbiotic and antagonistic roles of fungi within microbial communities are context-dependent (Jonsson et al., 2001; Heath and Tiffin, 2007; Yang et al., 2020). In the past, *Frankia* strains have demonstrated antagonistic effects against certain soil-borne pathogenic fungi such as *Fusarium solani* and *Cylindrocarpon destructans* on ginseng crops, suggesting that *Frankia* can inhibit fungal growth but not the other way around (Qi and Wang, 2021). To the best of our knowledge there are currently no reports of the microbes isolated in this study directly affecting or competing with *F. alni* as they have been shown here and the evidence from this study highlights their potential to do so.

As opposed to the effects of inoculating with microbes which had no effects on nodulation but led to lower biomass production in some instances where *F. alni* was co-inoculated, available N and P directly affected *F. alni* nodulation, underscoring their dual role as indicators of nutrient status and drivers of symbiotic investment. In line with our hypothesis, nitrogen availability correlated negatively with nodulation, with high N concentrations leading to an almost complete absence of nodules on the roots of *A. glutinosa*. These results are consistent with previous studies (Bélanger et al., 2011; Ballhorn et al., 2017) indicating that nitrogen-rich environments reduce a plant's reliance on nitrogen-

fixing symbionts as N demands can be met without an investment in symbiosis that is costly in carbon and energy (Chapin et al., 1987). Importantly, our results also highlight the available N-threshold at which nodule biomass declines. Although nodulation is almost completely absent after 24 × 15 ml applications of 20 mM NH_4NO_3 (total 210 mg of available N), a significant reduction in nodule biomass was already observed when using half of the concentration. This suggests that roughly 10 mM (total 105 mg of available N) marked the approximate N threshold where *A. glutinosa* trees have enough available N in the soil to either stop investing in their primary symbiont or where N directly inhibits *F. alni*. This value is notably higher than other similar fertilization experiments which used around 2.5 mM (Ballhorn et al., 2017), possibly owing to the poor initial concentration of N in the sterilized soil. However, it should be noted that even though 105 mg of available N is a very high amount of N, this was never present in the soil at once but rather supplied over 24 applications, with each application providing the soil with roughly 4.3 mg of N. This value that is much more indicative of forest field conditions which typically range between 0.5 and 5 mg/kg (Ren et al., 2021; Fu et al., 2024) while the 20 mM (8.6 mg supplied with each application) represents higher soil N concentrations in forest soils (Sun et al., 2022). Additionally, both NH_4^+-N and NO_3^--N were depleted in the pot soils at the end of the experiment for all concentrations, suggesting that despite the significant reduction in *F. alni* nodules, these N values were still within the range of what the growing trees could utilize.

Further in line with our hypothesis, increasing concentrations of available P led to an increase in both nodule biomass production and consequently in aboveground biomass production, consistent with the results of previous studies (Wall et al., 2000; Gentili and Huss-Danell, 2002; Valverde et al., 2002). This is likely because P influences the feedback mechanism regulating nodule growth, which is tied to the plant's nitrogen demand (Valverde et al., 2002). In general, supplying PO_4^{3-}-P to N-deficient soils (the concentrations in the soil that we used were NH_4^+-N: 38 ± 1.6 mg/kg soil; NO_3^--N: 15 ± 0.6 mg/kg soil) only resulted in enhanced biomass growth in the presence of *F. alni* and had no effect in their absence. Previous studies have interpreted this beneficial impact of P on the growth of symbiotic plants as a targeted effect on the regulation of nodule growth, rather than a broad enhancement of overall host plant growth (Valverde et al., 2002). However, when supplying trees with the highest concentration of P (which stimulated nodule growth in conditions of low available N) in combination with the highest concentration of N (which almost eliminated nodulation), P addition did not lead to nodule growth. Although past studies have assumed that available P is required for the initiation of nodulation in actinorhizal plants such as *Alnus incana* (Huss-Danell, 1997; Wall et al., 2000), our results suggest that this is not the case for *A. glutinosa* as the trees were still able to produce ample nodule biomass in the *F. alni*-inoculated control treatment and in the N-experiment where no additional P was supplied (soil PO_4^{3-}-P: 3.8 ± 0.4 mg/kg soil). This would explain why P-addition will neither initiate nor cause *A. glutinosa* to produce more biomass than what they are capable of producing with high N addition only (Gentili and Huss-Danell, 2003). Alternatively, it is also possible that the inhibitory effect of N on nodulation outweighs the stimulating effect of P. Under this assumption, the high P addition in our study may not have been sufficient to counteract the suppressive impact of high N. However, this interpretation contrasts with previous findings (Wall et al., 2000; Gentili and Huss-Danell, 2003), which showed that ammonium nitrate inhibited *A. incana* nodulation only at N/P ratios exceeding 7. Notably, in our study, the combined highest concentrations of N and P produced an N/P ratio of just 3, suggesting that for *A. glutinosa* this ratio might be lower than other actinorhizal plants. This relatively low soil N:P ratio contrasts with values reported for alder stands in the field, where N:P often ranges from 10 to 20 (e.g., Woś et al., 2022; Spohn and Stendahl, 2021). Soil N and P availability are not fixed properties but vary widely across landscapes due to differences in parent material, forest age, climate, and land-use legacies For example, P-rich soils derived from phosphate-bearing rock or those with a history of fertilization tend to show lower N:P ratios (Matkala et al., 2020), while older or N-fixing stands on P-limited substrates exhibit elevated N:P (Woś et al., 2022). As differences in N and P in sites are highly context dependent, further research is required to examine whether altering the N:P ratio in the soil would result in different effects on nodulation.

5. Conclusions

This study tested the different ways in which soil microbes from forests of different ages and N and P availability influence *F. alni* nodulation and consequently *A. glutinosa* tree performance. We found that while microbial communities derived from young and mature forests did not significantly influence nodulation when applied individually, mature forest fungal communities demonstrated the capacity to suppress the growth-promoting effects of *F. alni*. Although we only isolated part of the complex root-associated microbiome, we provide evidence of specific microbes such as *T. harzianum* and *Pseudomonas* sp. contributing to those suppressive effects. Furthermore, we highlight that nitrogen and phosphorus availability strongly and directly influence *F. alni* nodulation. High N concentrations significantly suppressed nodulation, establishing a threshold of available nitrogen beyond which *A. glutinosa* shifts away from its reliance on nitrogen-fixing symbiosis while increasing concentrations of P stimulated nodule biomass production in the absence of N addition. Overall, our findings emphasize the dominant role of nutrient availability in shaping the *A. glutinosa* - *F. alni* symbiosis, while also uncovering microbiome-specific interactions, such as fungi from mature forests and specific isolated microbes, that modulate the symbiosis.

CRediT authorship contribution statement

Konstantinos Georgopoulos: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **T. Martijn Bezemer:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Conceptualization. **Lars Vesterdal:** Writing – review & editing, Resources, Data curation. **Kaiyi Li:** Writing – review & editing, Investigation, Data curation. **Léon de Nobel:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Naksha Kasal:** Writing – review & editing, Investigation. **Sofia I.F. Gomes:** Writing – review & editing, Validation, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2025.106466.

Data availability

The data is deposited in Zenodo under (DOI): https://doi.org/10.5281/zenodo.16118454.

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