Afforesting with microbes: disentangling the effects of soil biotic and abiotic characteristics on trees using soil inoculation

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CHAPTER 3

Effects of soil biotic and abiotic characteristics on tree growth and aboveground herbivory during early afforestation

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Abstract

During early afforestation stages, biotic and abiotic soil characteristics change at different paces. However, the extent that each of these characteristics contribute to plant performance and subsequent herbivory remains unclear. This study aimed to study the effects of biotic and abiotic characteristics of forest soil on *Alnus glutinosa* performance and their subsequent impact on foliar herbivory during early afforestation. Soils were collected from a series of replicated forests of 10, 15 or 25 years old, planted in agricultural soils. Two experiments were conducted, focusing on the effect of soil microbiome (live vs. sterilized forest soil, and bulk sterilized soil vs. bulk inoculated with forest soil) and forest age on tree performance, root-associated microbial communities, and plant-herbivore interactions. In 10-year-old forest soil, *A. glutinosa* stems were thinner when grown in sterilized soil than when grown in live soil. In 15- and 25-year-old soil, trees exhibited lower fine root percentages and thicker stems in sterilized than in live soils, suggesting age-dependent responses possibly arising from plant and microbe nutrient competition. Overall root-associated microbial communities showed no significant differences in their composition based on forest ages. *Streptomyces* sp. and *Rokubacteriales* were differentially more abundant in the roots of trees growing in 15-year-old soils and their relative abundance was correlated positively with aboveground biomass, suggesting that effects of forest age on tree performance are contingent on the unique microbiome of each forest. The herbivory assay using *Mamestra brassicae* larvae revealed a positive correlation between leaf nitrogen content and leaf area consumption in the live vs. sterilized soil experiment, but not in the inoculated soil experiment. Trees in 10-year-old forest soils exhibited the highest herbivore performance, suggesting heightened herbivore susceptibility in early afforestation. Our findings underscore that intricate relationships between soil conditions, microbial communities, and plant-herbivore interactions affect tree performance and herbivory during early afforestation.

Keywords: Soil biotic characteristics, Soil abiotic characteristics, Afforestation, Herbivory, *Frankia alni, Alnus glutinosa*

1. Introduction

Afforestation is the process of establishing new forests at locations without prior tree cover. This process contributes greatly to increasing soil carbon (C) storage and can improve soil physiochemical and biological characteristics (Sun et al., 2016; Tedersoo et al., 2016; Kang et al., 2018; Asplund et al., 2019). Given that afforestation involves planting trees in a habitat that does not resemble a forest ecosystem, it is anticipated that changes in the biotic and abiotic characteristics of the soil will occur during the early stages of afforestation, shortly after tree planting (Ritter et al., 2003; Vopravil et al., 2021). As tree plantations mature, soil abiotic characteristics and microbial communities are also expected to change and follow a forest development trajectory (Koerner et al., 1999; Ritter et al., 2003; Liu et al., 2019b; Liu et al., 2020; De Marco et al., 2021). Soil microbial changes can occur rapidly, already within the first decade of afforestation, while abiotic conditions change slower and may start to stabilize several decades after tree planting (Gunina et al., 2017). These changes can have long-lasting impacts on the soil and on community structure as the forest matures (Gunina et al., 2017; Kang et al., 2018; Zhang et al., 2023). Consequently, it appears that soil abiotic and biotic characteristics change at different speeds during the early stages of afforestation, and there is limited knowledge about the contribution of each of these characteristics to tree performance.

Generally, soil pH and nutrient availability tend to decrease as forests age (Ritter et al., 2003) while soil organic matter increases (Fu et al., 2015; Wan et al., 2021). This is generally attributed to an increase in aboveground tree biomass and plant diversity leading to higher organic matter inputs (Waldrop et al., 2006; Eisenhauer et al., 2011). As trees mature, nitrogen (N) is continuously uptaken, incorporated into organic matter, and returned to the soil through leaf litter and other decaying organic material. This tight nutrient cycling reduces the availability of N in the soil (Rennie, 1955). When the initial pH is relatively high (i.e. in the case of afforestation of agricultural land), high organic matter input also leads to a reduction in soil pH due to nitrification occurring in the residue (Binkley and Richter, 1987; Rukshana et al., 2014; Binkley and Fisher, 2019). Given the slow pace at which abiotic characteristics change, it is crucial to evaluate their effects on tree performance during the initial stages of afforestation.

Throughout the succession of afforestation, soil biotic and abiotic characteristics increasingly resemble those of forest ecosystems (Gunina et al., 2017; Wu et al.,

2022; Guo et al., 2024) and soil microbial diversity increases as the composition of the microbial communities changes (Fu et al., 2015; Wan et al., 2021). The shift in microbial community composition is expected due to tree planting, which stimulates fungal community development and increases fungal diversity (Buckley and Schmidt, 2003; Jangid et al., 2011) through increased litterfall (Wang et al., 2018). As forests mature, trees shape fungal communities to a greater extent than bacterial ones, as many fungal taxa are associated with the roots of particular tree species, while many bacteria seem to be less tightly associated and their composition seems to be partly mediated by soil and litter chemistry (Urbanová et al., 2015). Consequently, the shaping of the bacterial community composition is partially attributed to changes in physical and chemical properties in the early successional stages (van der Wal et al., 2006; Yannikos et al., 2014; Liu et al., 2019a). However, many agricultural soils are dominated by bacteria (van der Wal et al., 2006) as practices like tillage and fertilization damage the hyphal network and negatively impact fungal root colonization (Helgason et al., 1998; Kahiluoto et al., 2001). During the bacteria-dominated early stages of afforestation on agricultural soils, N-fixing bacteria can play a crucial role in helping nitrogen fixing trees obtain N (Bélanger et al., 2011). An example of this is the ubiquitous actinobacterium *Frankia alni*, which symbiotically infects over 200 tree species, forming nodules in their roots and providing 70-90% of the hosts nitrogen needs via atmospheric fixation (Franche et al., 2009). Further, soil communities are expected to change from a predominance of bacteria to fungi as forests mature (Kang et al., 2018), suggesting that bacteria may have a stronger contribution than fungi for tree performance than fungi in the early stages of afforestation.

The effects of soil abiotic and biotic characteristics on trees also reflect changes in the chemical composition and nutritional quality of the foliage (Klepzig et al., 1995; Bélanger et al., 2011; Ballhorn et al., 2017). These changes likely affect tree susceptibility to foliar herbivores (Kos et al., 2015; Heinen et al., 2018; Huberty et al., 2020). An increase in soil N availability typically leads to increased N concentrations in leaves, which in turn increases their palatability to herbivores (Vandegehuchte et al., 2010). Soil microbial communities may also affect N uptake by trees, which could further influence herbivory (van Dijk et al., 2022). For instance, root colonization by plant growth promoting bacteria can lead to higher leaf protein content and therefore increase herbivory rates (Ballhorn et al., 2017). Yet, the extent to which beneficial microbial communities affect herbivory during these early stages

of afforestation remains unclear.

In this study we examined the effects of abiotic and biotic characteristics of forest soils on tree performance during the early stages of afforestation and assessed how these effects influence tree susceptibility to a generalist insect herbivore. To achieve this, we established two mesocosm experiments where tree performance was evaluated by growing trees of the species *Alnus glutinosa* in soils collected from nine afforested sites grouped into three age classes (10, 15 and 25 years old). In a first experiment, we compared live (reflecting abiotic properties and the presence of a live microbial community) and sterilized forest soils (reflecting abiotic properties and the absence of an alive microbial community). Meanwhile in a second experiment we inoculated 10% of forest soil (presence of an alive microbial community) into sterilized commercially obtained bulk soil (used to keep the abiotic characteristics similar across the mesocosms) and compared it to non-inoculated sterilized bulk soil to test the effects of biotic characteristics while keeping the abiotic characteristics constant. In addition, a herbivore performance experiment was conducted using leaves from both experiments. We hypothesized that biotic characteristics alone would lead to better tree performance than abiotic characteristics, at least in the earliest afforestation stage, and that the association with plant growth promoting bacteria would be the main driver of the relationship between the presence of a soil microbial community and improved *A. glutinosa* performance. We also expected that herbivory would be higher on trees grown in soils containing soil microbes (live and inoculated treatments), in particular those with higher relative abundance of plant growth promoting microbes.

2. Materials and methods

2.1 Forest soil sampling and tree seedlings

We selected nine tree plantations that were planted on former agricultural land in the provinces Drenthe and Groningen in the Netherlands. These plantations were initially planted with oak (*Quercus robur* and *Quercus petraea*) trees on loamy sandy soils, and all sites shared a similar crop cultivation history (SI, Table S1, S2). All forests were composed of multiple deciduous tree species, but were still dominated by oaks. Forests were planted between 1994 and 2012 and were categorized into three age classes (3 plantations of ~10-year-old, 3 of ~ 15-year-old and 3 of ~25-year-old). In March 2021 each forest was visited, and soil was collected from the top 30 cm layer

from three circular subplots (6 m in diameter) that were at least 20 m apart after removing the top litter layer. The soil samples from each subplot were kept separate and sealed in plastic bags for transportation.

In the laboratory, the collected soils were sieved through a 2 cm mesh to remove gravel, debris and large fauna and to homogenize the soil. Following the sieving, soil samples were kept for DNA analysis (stored at -20 °C). Soils were then stored at 4 °C for one week until further use. Soil properties were determined in independent subsamples of each sample. Soil moisture was measured by weighing the fresh and dry weight of the soil. Soil pH was measured using oven dried soil (1:1 soil:water ratio). Soil organic matter (SOM) was measured using the loss on ignition method (Heiri et al., 2001) by drying and weighing the soil first at 105 °C and then heating it to 550 °C in a muffle furnace. The soil heated at 550 °C was then used to determine the sand/silt fractions using a HAVER EML 200 Premium automated shaker (Oelde, Germany). Due to the very fine sieves used to measure silt and clay separately, soil texture was classified into % gravel (>2 mm), % sand (2 - 0.045 mm) and % silt and clay (<0.045 mm) (for soil properties, see SI, Table S1). Soil subsamples from each forest were oven dried (60°C) for nutrient analysis, including ammonium (NH_4), nitrate (NO_3) and orthophosphate (PO_4). NH_4 and NO_3 were determined using a 1M potassium chloride (KCl) extraction method (Kachurina et al., 2008) while PO_4 was determined using 0.01M calcium chloride (CaCl) as the extraction reagent (Houba et al., 2008). The final concentrations were calculated in mg NH_4, NO_3 and PO_4 per kg of soil (See SI, Table S3).

We selected black alder (*Alnus glutinosa,* (L.) Gaertnr) as test tree species. Despite the clear dominance of oak (*Quercus robur* and *Quercus petraea*) trees, *A. glutinosa* trees were present in all sampled sites. Native alder seeds were obtained from the Nature Agency Staatsbosbeheer in the Netherlands.

2.2 Mesocosm experiments

Two experiments were established in an attempt to disentangle the effects of forest soil abiotic and biotic characteristics on *A. glutinosa* growth. In the first experiment, we compared the effect of live forest soils and that of sterilized forest soils. It is important to note that the live soils represent the combination of biotic and abiotic characteristics, while sterilized soils only reflect the abiotic characteristics. However,

sterilization can also lead to the release of nutrients and to a reduction in pH and microbial biomass carbon (Salonius et al., 1967; McLaren, 1969; Thompson, 1990; Dietrich et al., 2020). To account for that, we established a second experiment, in which sterilized commercial bulk soil was inoculated with the live forest soil. Inoculation of live soil into sterilized bulk soil reflects the biotic characteristics while abiotic conditions are kept relatively unchanged (for the bulk soil properties, see SI, Table S1). By using the same sterilized bulk soil across all pots that received the soil inoculum, we aimed to keep the effects of nutrient release and affecting abiotic soil conditions similar across all the mesocosms in the second experiment. Thus, we assumed that any potential difference observed between trees grown under the live treatment of experiment 1 and the inoculated treatment of experiment 2 would be attributed to the effects the soil abiotic characteristics.

2.2.1 Experiment 1: live vs sterilized forest soils

We compared the performance of *A. glutinosa* grown in live and sterilized forest soils belonging to the three age classes. All soils were sterilized by autoclaving at 120 °C for 1 h. Pots (3.15 L, 10 x 10 x 12 cm) were filled with 3000 g of live or sterilized soils from each of the three subplots of the nine forests (N = 9 forests x 3 subplots x 2 treatments = 54 pots). Each soil sample was duplicated, resulting in 108 pots. The data collected from the two pseudo-replicates was averaged to obtain one value per subplot for all variables measured below.

A.glutinosa seeds were surface sterilized using commercial sodium hypochlorite solution and were then germinated on autoclaved soil to avoid microbial contamination. The seeds were germinated in a growth chamber at 70% relative humidity, a light regime of 16h:8h (light:dark), and air temperature of 20 °C (light) and 18 °C (dark). One two-week-old *A. glutinosa* seedling with two developed leaves (~2.5 cm stem height) was transferred in each pot. The pots were placed in a growth chamber under the same conditions. Seedlings were watered three times per week and replaced during the first week in case of death (only 4 seedlings were replaced). Eighteen weeks after transplantation, seedling height and stem diameter were recorded, and plants were harvested. For each pot, roots were washed under running tap water and, the number of nodules was recorded to obtain the root nodule density. Following that, root subsamples were taken for morphological characterization (stored in water at 4 °C until performed scans) and DNA sequencing

(stored at -20 °C until DNA extraction). The root subsamples for morphological characterization were scanned with an Epson Perfection V850 Pro scanner, with 1200 dpi, and analyzed with the WinRHIZO software (Regent Instruments, Quebec, QC, Canada) to determine root length, specific root length (root length / dry root biomass) and percentage of fine roots (we consider as diameter < 0.3 mm). To determine the root associated microbiome, the samples were not surface sterilized as we were interested in both the endophytes and the microbes on the roots surface for a more complete characterization of the root associated microbiome. DNA analysis was done only for the live treatment because we wanted to compare the effects of the forest microbiomes of live and inoculated treatments. While we recognise that after the initiation of the experiment, sterile soils will certainly be colonised, only the live soils were sequenced because microbial communities in the sterilized mesocosms are a result of random colonisation which is not informative to our work. Stems and roots were oven dried, above and belowground dry biomass was determined and the root:shoot ratio (dry root biomass / dry shoot biomass) was calculated. To analyse leaf N content, oven dried soil subsamples were ground on a QIAGEN TissueLyser II Bead Mill (Hilden, Germany) at 370 rpm for 5 min. Subsequently, the dry combustion method (Matejovic, 1997) was used to measure leaf N percentage using a Thermo Scientific FLASH 2000 CN analyser (Milano, Italy).

2.2.2 Experiment 2: 10% inoculation of forest soils

The second experiment focused on the effects of the soil biotic characteristics. For this scope, a standard “whole-soil inoculum” method was used (Ma et al., 2020; Pangesti et al., 2020; Pineda et al., 2020). Sterilized bulk soil was inoculated with 10% of the different forest soils (2700 g bulk soil and 300 g forest soil). The bulk soil was collected from an agricultural grassland and sterilized by drying at 80 °C for 36 hours, followed by microwaving at 900 Watt for 5 minutes (Alessio et al., 2017). The bulk soil was microwaved and not autoclaved due to technical difficulties with the autoclave (See SI, Fig. S1 for efficiency comparison between both methods). A total of 54 pots of 3.15 L were filled with inoculated soils from the three subplots per forest, in duplicate. An additional ten control pots were filled with sterilized bulk soil only. The control pots were ten instead of nine to ensure a sufficient number of surviving replicates in the autoclaved bulk soil of our control group while maintaining statistical power. To address the unbalanced design caused by this, linear mixed effects (LMM) and generalized linear mixed effects (GLMM)

models were used (see section 2.6 below) which are well suited for controlling the potential biases arising from the unbalanced replication. Plants were grown under the same conditions as described for Experiment 1 for 18 weeks, after which they were harvested as described for experiment 1 and the same plant performance parameters were measured.

2.3 Herbivory assay

In the week before the harvest of both experiments, the 6th youngest leaf from each tree (6th leaf from the top) was removed and used in a bioassay that aimed to assess the effect of forest soil biotic and abiotic characteristics on herbivory. The petiole was placed in an Eppendorf tube filled with wet cotton to prevent dehydration of the leaf, and then sealed with parafilm. Caterpillars of the phytophagous species *Mamestra brassicae* (cabbage moth; Lepidoptera: Noctuidae (Rojas et al., 2000)) were used in this study. The larvae of the cabbage moth are considered generalists and feed on a wide range of plant hosts including on several deciduous tree species such as oak and willow, and occasional feeding on *A. glutinosa* leaves has been observed (Carter, 1984). Eggs of a population collected from cabbage fields near Wageningen university were obtained from the Department of Entomology at Wageningen University, and the population was reared on *Brassica oleracea* var. *gemmifera* cv. Cyrus (Zhu et al., 2018) prior to their use. One second instar caterpillar was added to each leaf in a 500 ml transparent container after a starvation period of 24 h. The containers were placed in a climate chamber at a photoperiod of 16:8 (light:darkness) and a temperature regime of 25 °C (light) and 20 °C (dark). After five days, caterpillars were removed from the containers. Larval initial and final weight were recorded and used to calculate weight gain during the feeding period. Leaves were then scanned, and total area consumed was estimated using ImageJ v1.53s.

2.4 Soil and root-associated bacterial and fungal communities

Forest soil DNA was extracted using the DNeasy PowerSoil pro kit (Qiagen Inc., Hilden, Germany). DNA from root samples from the live treatment of Experiment 1 and from Experiment 2 was extracted using the DNeasy plant Pro kit (Qiagen Inc., Hilden, Germany) following the protocols of the manufacturer. We used the indexed primers 515F (GTGYCAGCMGCCGCGGTAA) and 926R (GGCCGYCAATTYMTTTRAGTTT) (Quince et al., 2011; Parada et al., 2015)

to target the V4 region of the 16Sr RNA gene of bacteria, and primers gITS7ngs (GTGARTCATCRARTYTTTG) and ITS4ngsUni (CCTSCSCTTANTDATATGC) (Tedersoo and Lindahl, 2016) to target the rRNA ITS2 region of fungi. All polymerase chain reactions (PCR) were conducted in duplicate. To confirm PCR success, 5 µl of PCR products were subjected to 1% agarose gel electrophoresis. Both a negative control (comprising ddH_2O with no DNA template) and a positive control sample (consisting of an artificial DNA molecule with multiple primer sites) were used to assess potential contamination during sample preparation for PCR and to evaluate the efficiency of the PCR process, respectively. Sequencing was performed with NovaSeq6000 at NovoGene.

Given the symbiotic association between *A. glutinosa* and *F. alni*, in addition to sequencing fungal and bacterial communities, we quantified *F. alni* presence in the initial soils. This step aimed to ascertain whether root nodule density was dependent on the initial presence in the different forest soils. For that, we performed real-time PCR (qPCR) using a CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA). Quantification of *F. alni* using qPCR was carried out for each of the three soil samples per forest site. We selected the primer combination 23Fra1655f/23Fra1769r (targeting 133 bp of 23S rRNA) to detect the N-fixing Frankia strains in the soil samples (Samant et al., 2014). The reaction mixture, with a total volume of 10 µl, consisted of 5 µl of iQ SYBR Green Supermix (BioRad, Hercules, CA), 0.2 µl of each primer (100 nM each), and 1 µl of the template. The qPCR involved an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C, annealing at 64 °C and extension at 72 °C, each step lasting 30 s. A melting curve analysis was performed after the amplification. Purified PCR products from *Frankia alni* nodules of tree roots were used to generate standard curves for quantification after measuring their concentrations using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

2.5 Bioinformatics

High-throughput sequencing (NovaSeq) produced 10,685,252 and 14,182,206 raw paired-end sequences for 16S (bacteria) and ITS2 (fungi) libraries, respectively. Raw sequencing data was processed using PipeCraft2 (v1.0.0) bioinformatics platform for metabarcoding data (Anslan et al. 2017). Sequences were demultiplexed (within demultiplexing module) using cutadapt v3.5 (Martin, 2011), which resulted in an

average of 128,959 and 125,504 sequences per sample for 16S and ITS2 libraries, respectively. Demultiplexed sequences were quality filtered (maximum number of expected errors per sequence = 2 and discard sequences with any ambiguous base) and denoised and assembled with default settings utilizing DADA2 v1.20 (Callahan et al., 2016) within PipeCraft2. Denoised 16S sequences were subjected to chimera filtering using the "consensus" method of DADA2, after which, the average number of sequences per sample was 92740. Taxonomy was assigned to the resulting amplicon sequence variants (ASVs) with DADA2 classifier (with default settings) against Silva v138.1 database (Quast et al., 2013).

Quality filtered, denoised and assembled ITS2 ASVs (average of 93,747 sequences per sample) were subjected to ITSx v1.1.3 (Bengtsson-Palme et al., 2013) to extract fungal ITS2 region without flanking 5.8S and 28S genes (primer binding sites). Putative chimeras were removed with uchime denovo and uchime reference based methods (Edgar et al., 2011) in PipeCraft2 using UNITE v9 (Abarenkov et al., 2022) database as a reference. The resulting dataset consised on average of 93,109 sequences per sample. Since an individual fungal genome may host multiple different ITS copies (Hakimzadeh et al., 2023) we further clusterd ITS2 ASVs to operational taxonomic units (OTUs) based on 97% similarity threshold using vsearch 2.23.0 (Rognes et al., 2016); within the "ASV to I" module of PipeCraft2 (similarity type, i.e. --iddef = 2, other settings as default). Additionally, OTUs were post-clustered using the LULU v0.1.0 algorithm (Frøslev et al., 2017) with minimum_match = 90 (other settings as default) to merge consistently co-occurring 'daughter-OTUs'. Taxonomic assignment of the OTUs was done using BLAST 2.11.0+ (Camacho et al., 2009) (task = blastn, evalue = 0.001, word_size = 7, reward = 1, penalty = -1, gapopen = 1, gapextend = 2) against UNITE v9 database (Abarenkov et al., 2022). The FungalTraits database was used to assign fungal guilds to each fungal OTU (Põlme et al., 2020). Based on taxonomy assignment results, we removed all bacterial ASVs that did not belong to the kingdom: Bacteria and the ones that belonged to the Chloroplast order and the Mitochondria family. We also removed all fungal OTUs that did not belong to the kingdom: Fungi.

2.6 Statistical analysis

2.6.1 Plant performance

In experiment 1, a linear mixed effects model (LMM; n = 9) was used to test the interaction between soil treatment (live vs sterilized) and forest age (10-year-old, 15-year-old, 25-year-old) on each of the plant performance parameters (see SI, Table S4) measured individually with the *lme4* R package v1.1-35.1 (Bates et al., 2015). We considered forest identity (i.e. a unique number given to each individual forest, see SI, Table S1) as a random factor to account for the non-independence between the three subplots within each forest. For proportion data like root to shoot ratio, the percentage of fine roots and the percentage leaf N, a generalized linear mixed effects model (GLMM) was used. For root nodule density, a GLMM was used with negative binomial distribution due to the overdispersion of data using the *performance* R package v2.0.4 (Lüdecke et al., 2021).

In experiment 2, we first assessed whether inoculation influenced tree performance as compared to sterilized bulk soil (control), and then we tested for differences between the three forest ages (n = 9). To assess the effect of inoculation, we used an individual LMM with each of the measured plant performance variables (except for root to shoot ratio, the percentage of fine roots, the percentage leaf N and root nodule density where GLMM was used, as done in Experiment 1) as response variables, and forest age as the main factor (see SI, Table S5, S6). For this purpose, the control was included as one of the levels within forest age. Forest identity was used as a random factor similarly as described above for experiment 1. The control was included as a category in forest age and was added as part of the forest identity (random factor) to account for non-independence like the forest subplots. If the LMM output was significant, the effect of forest soil inoculation was tested with a Dunett's comparison test to compare each of the three age classes against the control, using the *DescTools* R package v0.99.51 (Signorell et al., 2023). To assess the effect of forest age, the controls were excluded and an additional LMM was used to test the effect of forest age on each of the plant performance parameters. Forest identity was used as a random factor. For all models, the normality of the residuals and their distribution was assessed using a Shapiro-Wilk normality test and a qqplot, respectively. Additionally, a histogram was used to visually assess the skewness of the data. Homogeneity of variance between samples was tested using a Levene's test. Specific root length of experiment 1 and the weight gained by the

caterpillars in experiment 2 were square-root transformed to improve data normality. Potential correlations between root nodule density and plant performance or leaf N were evaluated with Pearson correlations using the *Performanceanalytics* R package v2.0.4 (Peterson et al., 2020).

2.6.2 Microbiomes

After bioinformatics, the soil and root sample data sets were treated independently. To normalize sample sequencing depth prior to analysis, the tables of bacterial ASVs and fungal OTUs were rarefied to 17,765 and 1,253 reads for roots and 34,858 and 17,583 reads for soils respectively based on the sample with lowest sequencing depth for each dataset. We removed ASVs and OTUs with low abundance (represented by less than 0.01% of total reads). Furthermore, ASVs and OTUs that appeared in less than 3 of the samples were filtered out. To visualize differences in the community structure between forest ages, we performed a principal coordinate analysis (PCoA) based on the Bray-Curtis distances on the square root counts calculated using the *ordinate* function of the *phyloseq* R package v1.46.0 (McMurdie and Holmes, 2013). A Permanova analysis was performed using the *vegan* R package v2.6-4 to assess differences in the bacterial and fungal communities between different forest ages (Dixon, 2003). Overdispersion of data was assessed to check whether the results were not because of the different ingroup variation using the *DEseq2* R package v1.42.0 (Love et al., 2014). The *pairwiseAdonis* R package v0.4.1 was used to perform pairwise comparison tests when the Permanova results were significant (Martinez Arbizu, 2020). Finally, to identify the key taxa associated with each forest age, we used the relative abundances of each ASV/OTU and performed a linear discriminant effect size analysis (LEfSe) (Segata et al., 2011) using the Galaxy web application (Afgan et al., 2022). This analysis makes use of non-parametric factorial Kruskal-Wallis sum-rank test to detect taxa with significant differential abundance based on each age group. It then uses a Wilcoxon rank-sum test to perform pairwise tests and linear discriminant analysis to estimate the effect size of each differentially abundant taxa. An ASV/OTU was considered unique for an age class when more than 75% of the ASV/OTU reads belonged to that specific age class (i.e.10-year-old, 15-year-old, 25-year-old).

2.6.3 Herbivory

In Experiment 1, two LMMs were used with treatment and forest age as main factors,

and either the total leaf area eaten, or larval weight gain as response variables. If a significant effect was found, individual comparisons were done by means of a Tukey's test. In Experiment 2, the effect of inoculation on total leaf area eaten or larval weight gain was assessed using a LMM in which forest soil age was the main factor. For this purpose, the control was included as factor level of age. If a significant effect was found, the effect of inoculation was assessed independently for each forest soil age by comparing herbivory in trees grown in inoculated vs control soil based on a Dunett's test. To assess the effect of forest age, the controls were then excluded and an additional LMM was used to test the effect of forest age on area eaten and weight gained by the caterpillars. For both experiment one and two, we also evaluated whether total leaf area eaten and larval weight gain were correlated with the percentage of total leaf N and nodule density by means of linear regressions.

All statistical analyses outlined above were performed in R v4.2.1 and Rstudio v1-554 for windows (R Core Team, 2022). All graphs were made using the *ggplot2* R package v3.4.4 (Wickham, 2011).

2.7 Accession numbers

The raw Illumina reads are deposited in European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB72733, (BioSamples SAMEA115336815 - SAMEA115336894; Accessions ERS18334618 - ERS18334697).

3. Results

3.1 *Alnus glutinosa* tree performance

3.1.1 Experiment 1: live vs sterilized forest soils

Significant interactions were detected between forest age and soil sterilization for stem width, specific root length, percentage of fine roots and nodule density (Fig. 1; SI, Table S4). Percentage of fine roots did not differ between 10-year-old live and sterilized soils, but there were fewer fine roots in sterilized soils than in live soils for trees growing in 15- and 25-year-old soils (Fig. 1F; SI, Table S4). Although differences were not significant between the live and sterilized treatments, trees growing in sterilized soils had a lower average stem width compared to trees growing in live soils for the 10-year-old soils, while the opposite was observed for 15- and

25-year-old soils (Fig. 1B; SI, Table S4). These results suggest that removing the biotic characteristics has differential effects among forest ages for stem width and percentage of fine roots. In 15- and 25-year-old soils trees seem to be affected in a similar manner, therefore having a different effect from the 10-year-old soils. Nodule density was significantly higher in live than in sterilized soils for 10- and 15-year-old soils and as expected was almost absent in sterilized soils (SI, Table S4). However, in 25-year-old live soil, the root nodule density was low and it was indistinguishable from that of roots in 25-year-old sterilized soil. Forest age also significantly influenced nodule density, so that nodule density decreased with increasing forest age (Fig. 1H; SI, Table S4). Root nodule density was not dependent on the abundance of *F. alni* originally present in the forest soils (SI, Fig. S2B). Interestingly however, *A. glutinosa* trees growing in live 10-year-old soils had more than twice the density of nodules than trees growing in live 15-year-old soils and five times the nodule density of trees growing in live 25-year-old soils.

In live soils, root nodule density was positively correlated with percentage leaf N (R^2 = 0.29, p = 0.010; SI, Fig. S3) but negatively correlated with belowground biomass (R^2 = 0.15, p = 0.040; SI, Fig. S4B) and root to shoot ratio (R^2 = 0.28, p = 0.010; SI, Fig. S4D).

There was no effect of age on the initial soil NH_4, NO_3 and PO_4 levels. Despite the lack of a significant effect of age, all three nutrients displayed an increase of average concentration with increasing forest age (particularly NO_3 and PO_4), suggesting higher N and P levels as forests develop during early afforestation (SI, Fig S5A, B, C). There were no correlations between any of the soil nutrients and either above/belowground biomass production or nodule density.

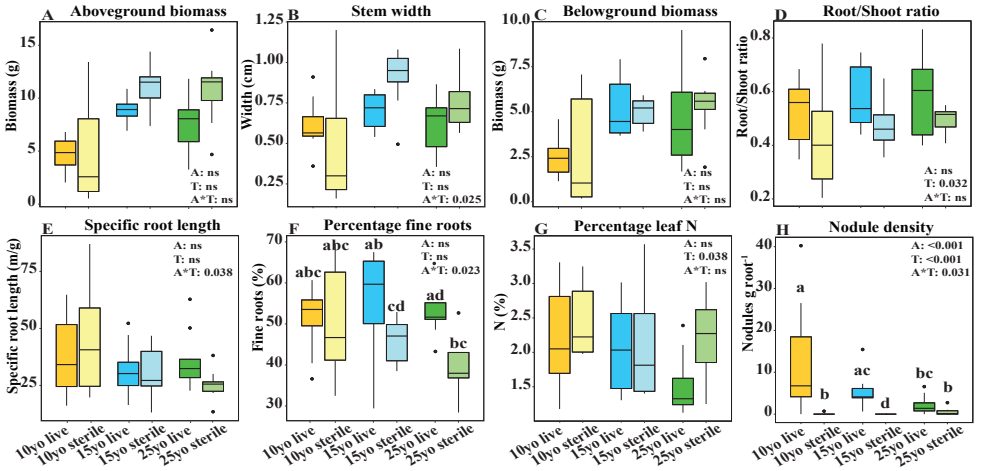

Figure 1 | The effect of forest Age (A; 10yo, 15yo, 25yo), treatment (T; live, sterilized soil) and their interaction (A*T) on (A) Aboveground biomass, (B) Stem width, (C) Belowground biomass, (D) Root/Shoot ratio, (E) Specific root length, (F) Percentage fine roots, (G) Percentage leaf N, and (H) Nodule density. The vibrant colours represent the live soil treatments and the faded colours the sterilized soil treatments for each age group. Whiskers represent the 95 % confidence interval with the bottom and top horizontal lines of the box being the 25th and 75th percentiles respectively. The line inside the box represents the median. These were calculated from n = 9 replicates. Statistical output of the full linear mixed effects model is also presented. Details can be found in Table S4. Different letters

3.1.2 Experiment 2: 10% inoculation of forest soils

Forest soil inoculation had a significant effect (compared to bulk sterilized soil) on multiple measured parameters of tree performance, so that trees growing in inoculated soils had lower stem width, lower belowground biomass, and lower root:shoot ratio, and a higher percentage of leaf N and root nodule density (Fig. 2; SI, Table S5). Within the three forest ages, the linear mixed effect models showed that the only plant performance parameter significantly affected by forest age was aboveground biomass, even though the post-hoc test did not indicate differences between specific ages (SI, Table S6). Yet, trees grown in soil inoculated with 15-year-old forest soil produced 26% more aboveground biomass than those inoculated with 10-year-old and 36% more than those inoculated with 25-year-old forest soil (Fig. 2A; SI, Table S6). These results show that only aboveground biomass was affected by the biotic characteristics of the forest soils with different ages.

Despite the non-significant effect of forest soil age on root nodule density, we observed a higher number of root nodules per gram root in trees grown in soil inoculated with 10 and 15-year-old forest soil than in those growing in soils inoculated with 25-year-old forest soil, indicating a reduction in nodule density in the older soils (Fig. 2H; SI,

Table S6). The same was true for the percentage of leaf N (Fig. 2G; SI, Table S6). Root nodule density was negatively correlated with root:shoot ratio ($R^2 = 0.17$, $p = 0.030$; SI, Fig. S6D) but positively with percentage leaf N ($R^2 = 0.46$, $p < 0.001$; SI, Fig. S7A) which in turn was positively correlated with aboveground biomass ($R^2 = 0.17$, $p = 0.030$; SI, Fig. S7B). There was no significant correlation between root nodule density and the inoculum of *F. alni* present in the forest soils (SI, Fig. S8).

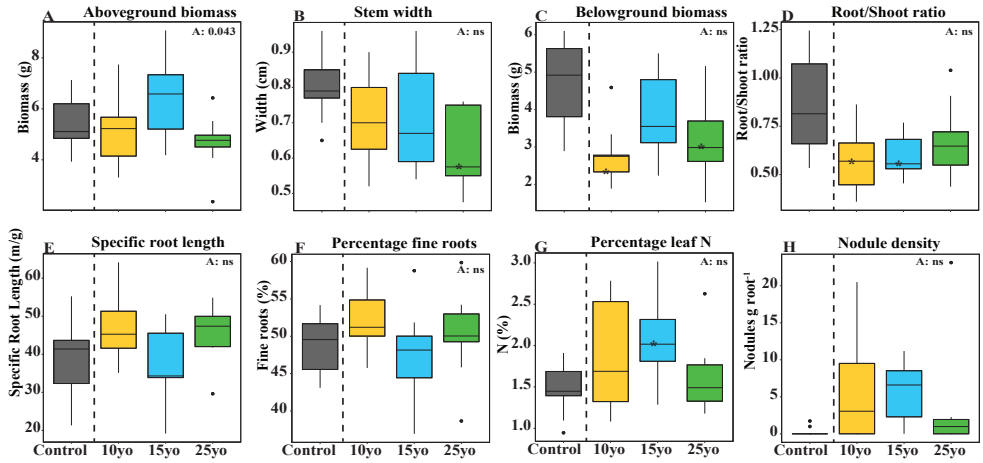

Figure 2 | Effect of forest Age (A; 10yo, 15yo, 25yo) on (A) Aboveground biomass, (B) Stem width, (C) Belowground biomass, (D) Root/Shoot ratio, (E) Specific root length, (F) Percentage fine roots, (G) Percentage leaf N, and (H) Nodule density. Whiskers represent the 95 % confidence interval with the bottom and top of the box being the 25th and 75th percentiles respectively. The line inside the box represents the median. These were calculated on values from n = 9 replicates. Statistical output of the full linear mixed effects model where ages are compared including the Control can be found in Table S5. An asterisk inside a boxplot indicates significant differences from the control treatment indicated by a Dunett's test following a significant LMM output. Significance of age is reported based on a LMM between only the three age groups. Statistical output of the full linear mixed effects model where only ages are compared without the Control can be found in Table S6.

3.2 Microbial community composition

3.2.1 Soil microbial community composition

In total, 3265 bacterial ASVs and 1126 fungal OTUs were detected in the in the field collected forest soils. Of these, 1326 bacterial ASVs and 225 fungal OTUs were shared between the three age groups. For bacteria, 269, 216 and 185 bacterial ASVs were unique to the soils from 10, 15 and 25-year-old forests respectively, while for fungi, 200, 170 and 160 were unique to each respective age group (SI, Fig. S9E, F). In the field collected soil, both bacterial (Permanova: pseudoF = 3.24, $R^2 = 0.21$, $p < 0.001$) and fungal (Permanova: pseudoF = 3.11, $R^2 = 0.22$, $p < 0.001$) community composition significantly differed among forest ages (SI, Fig. S9A, B). However,

no differences were identified in soil bacterial or fungal richness (SI, Fig. S9C, D).

3.2.2 Experiment 1

In total, 477 bacterial ASVs and 54 fungal OTUs were detected in the roots of trees growing in the live soils. Of these, 117 bacterial ASVs and 4 fungal OTUs were shared between the three age groups. For bacteria, 48, 68 and 27 bacterial ASVs were unique to trees growing in 10, 15 and 25-year-old forest soil, respectively, while for fungi, 8, 12 and 6 were unique to each respective age group (Fig. 3A, D).

There were no differences in microbial community composition in the roots of trees grown in the three different forest ages for bacteria (Fig. 3B; Permanova: pseudoF = 1.27, $R^2 = 0.09$, $p = 0.150$) or fungi (Fig. 3E; Permanova: pseudoF = 1.20, $R^2 = 0.09$, $p = 0.100$). Neither were there differences in bacterial or fungal richness (SI, Fig. 10A, C) or in the number of root-associated bacterial phyla or fungal classes among trees grown in the three different forest ages (SI, Fig. S11).

Linear discriminant effect size analysis (LEfSe) revealed that *Actinobacteria*, *Methylococcaceae, Rokubacteriales, Burkhoderiales, Caulobacteriales, Sphingomonadaceae, Microtrichales, Planctomycetes, Nostocaceae, Micrococcales, Gemmatimonadota, Myxococcota, Streptomyces* sp.*, Pseudomonadales, Rhizobiales, Mycobacterium, Nitrospiraceae* and *Pleosporales* were differentially more abundant in the roots of trees growing in 15-year-old soils (Fig. 3A, D; SI, Fig. S12, S13; for classification up to genus level see SI, Table S7, S8). *Streptomyces* sp. and *Rokubacteriales* were positively correlated with aboveground biomass and *Streptomyces* sp. were negatively correlated with specific root length (SI, Table S9). There was no correlation between any fungal OTUs and any tree performance or leaf herbivory variables.

Despite the presence of unique bacterial ASVs and fungal OTUs in the roots of trees growing in the 10- and 25-year-old live forest soil, none of the taxa they corresponded to were differentially more abundant (Fig. 3A, D).

Lastly, we tested whether forest age impacted the relative abundance of common fungal symbionts found in the roots of *A. glutinosa*, like *Endogonomycetes* and major fungal guilds like ectomycorrhizal (EMF) and pathogenic fungi and whether the relative abundance of these fungi affected the biomass production and nodulation of *A. glutinosa*. There were no significant differences in the relative abundance of

EMFs and pathogenic fungi in the roots of trees growth in the three different ages (SI, Fig. S14A, C) and *Endogonomycetes* were not detected in the analysis.

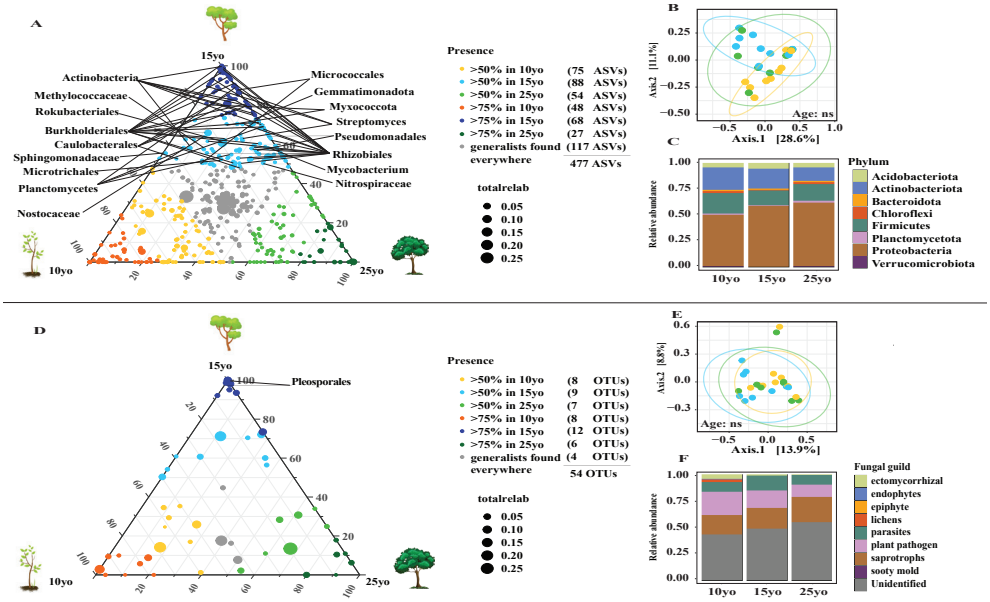

Figure 3 | Ternary plots of bacterial ASVs (A) and fungal OTUs (E) present in at least three samples with a relative abundance higher than 0.01 %. The colour represents the age where it is primarily found based on the total number of reads. Highlighted taxa are significantly differentially more abundant taxa for a single age class, informed by the LEfSe analysis (see Figs. S10 and S11 for details of the analysis). Bacterial (B) and fungal (F) community composition (based on Bray-Curtis similarity) and alpha diversity (C, G); letters indicate significant differences. Taxa bar plots showing the relative abundance of the eight most abundant bacterial phyla (D) and the fungal guilds (H) present at each forest age (10yo, 15yo, 25yo).

3.2.3 Experiment 2

In total, 482 bacterial ASVs and 61 fungal OTUs were detected in the roots of trees growing in soil inoculated with forest soils. Of these, 170 bacterial ASVs and 4 fungal OTUs were shared between the three age groups. For bacteria, 35, 24 and 31 bacterial ASVs were unique to trees growing in 10, 15 and 25-year-old forest soil, respectively, while for fungi, 5, 11 and 11 were unique to each respective age group (Fig. 4A, D).

Bacterial community composition in the roots did not differ between the three different forest ages (Permanova: pseudoF = 1.37, $R^2 = 0.11$, p = 0.100) (Fig. 4B). However, when only inoculated soils were compared, forest age significantly affected fungal root-associated community structure (Permanova: pseudoF = 1.69, $R^2 = 0.13$, p = 0.004) (Fig. 4E). A pairwise comparison test revealed a significant difference

between the root-associated fungal communities of trees grown in soil inoculated with 15-year-old forest soil and trees grown in soil inoculated with 25-year-old forest soil ($F = 2.16$, $R^2 = 0.13$, $p = 0.007$). Bacterial ASV or fungal OTU richness (SI, Fig. S10B, D) and the number of root-associated bacterial phyla or fungal classes did not differ significantly among the age groups (SI, Fig. S15A, B).

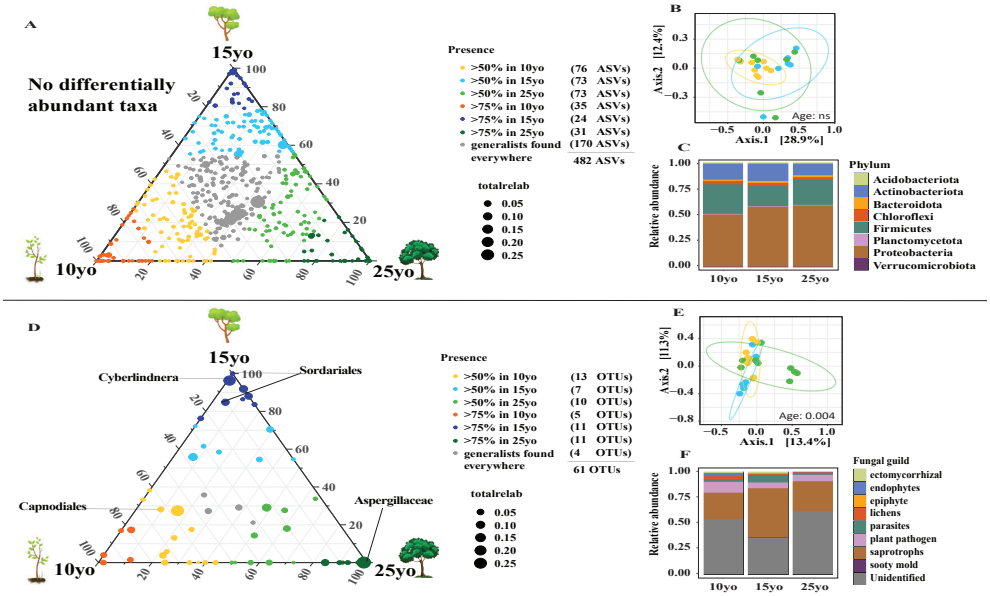

Figure 4 | Ternary plots of bacterial ASVs (A) and fungal OTUs (E) present in at least three samples with a relative abundance higher than 0.01 %. The colour represents the age where it is primarily found based on the total number of reads. Highlighted taxa are significantly differentially more abundant taxa for a single age class, informed by the LEfSe analysis (see fig. S13 for the details of the analysis). Bacterial (B) and fungal (F) community composition (based on Bray-Curtis similarity) and alpha diversity (C, G); letters indicate significant differences. Taxa bar plots showing the relative abundance of the eight most abundant bacterial phyla (D) and the fungal guilds (H) present at each forest age (10yo, 15yo, 25yo).

Linear discriminant effect size analysis (LEfSe) revealed that *Capnodiales* were differentially more abundant in the roots of trees growing in 10-year-old soils while *Cyberlindera* and *Sordiales* were differentially more abundant in 15-year-old soils and *Aspergilaceae* in 25-year-old soils (SI, Fig. S16; for classification up to genus level see SI, Table S10). Despite the presence of unique bacterial ASVs, no bacterial taxa were found to be differentially more abundant for the three ages (Fig. 4A). No significant correlation was detected between any of the bacterial ASVs or fungal OTUs and any tree performance or leaf herbivory variables.

Similarly to the first experiment, we tested whether forest age impacted the relative

abundance of *Endogonomycetes* EMFs and pathogenic fungi and whether the relative abundance of these fungi affected the biomass production and nodulation of *A. glutinosa*. There were no significant differences in the relative abundance of EMFs and pathogenic fungi in the roots of trees growth in the three different ages (SI, Fig. S14B, D) and *Endogonomycetes* were not detected in the analysis.

3.3 Mamestra brassicae performance

In experiment 1 (live vs sterilized forest soils), age and sterilization did not affect the total leaf area eaten by *M. brassicae* larvae or weight gain (Fig. 5A, B). However, a positive correlation was observed between the percentage leaf N and both total leaf area eaten ($R^2 = 0.13$, $p = 0.020$; Fig. 5C) and larval weight gain ($R^2 = 0.33$, $p<0.001$; Fig. 5D).

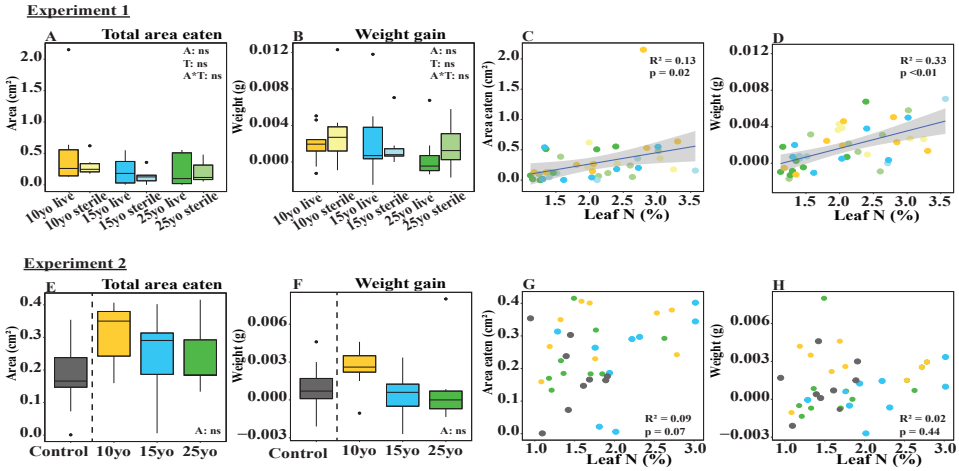

Figure 5 | The effects of forest age (10yo, 15yo, 25yo) and treatment (Live, Sterilized) in experiment 1 (1) and the effect of forest Age (10yo, 15yo, 25yo) in experiment 2 (2) on (A, E) leaf total area eaten and (B, F) Mamestra brasicae weight gain and the correlation between percentage leaf N and leaf area eaten (C, G) and Leaf N% and Mamestra brasicae weight gain (D, H) in both experiments. The error bars represent the 95 % confidence interval with the bottom and top of the box being the 25th and 75th percentiles respectively. The line inside the box represents the median.

In experiment 2, in the analyses where both inoculated and control were included, inoculation did not significantly affect leaf consumption or larval weight gain. Leaf consumption appeared higher in inoculated treatments, whereas larval weight gain appeared higher only in 10-year-old soils (Fig. 5E, F). The same was observed when comparing among inoculated soils (without the sterilized bulk control). Additionally, there was no correlation with percentage leaf N (Fig. 5G, H). The abundance of *F.*

alni in the forest soils was not correlated with leaf area eaten or caterpillar weight gain in either of the experiments (SI, Fig. S17).

4. Discussion

This study examined the effects of soil biotic and abiotic characteristics and their impact on alder tree performance during early stages of afforestation, and its subsequent effect on a generalist herbivore that feeds on leaves of those trees. Our findings highlight the significant influence of soil microbiomes on plant growth. Furthermore, we reveal that key bacterial taxa, besides *F. alni*, play an important role in promoting tree growth, regardless of forest age.

In partial contrast to our initial hypothesis, sterilizing soil and hence, the absence of a soil microbiome, did not consistently decrease tree performance. The impact of removing the soil microbiome on tree performance varied with forest age. In 10-year-old soils, stem width was significantly and negatively impacted by soil sterilization. In contrast, trees grown in the older soils (15- and 25-year-old) had thicker stems when the soil microbiome was removed by sterilization and they exhibited a lower percentage of fine roots, while no differences in the percentage of fine roots were found when growing in the 10-year-old soils. The differential effect of soil microbiome presence on *A. glutinosa* performance based on the age of the forest soil could be due to less competition with microbes for nutrients in sterilized compared to live soils. In older forest soils, where SOM is higher, microbes can rapidly take up more N compared to roots after its mobilization from SOM and litter (Kuzyakov and Xu, 2013). Thus, removing the microbiome in older soils may reduce competition for nutrients, resulting in increased biomass and thicker stems. Additionally, the reduced necessity for trees to invest in fine roots for nutrient scavenging and competition with microbes could explain the observed decrease in fine root percentage in sterilized soils. It cannot be excluded that the sterilization treatment could have led to the release of nutrients, leading to soils with higher quantities of readily available nutrients, which could have allowed trees to invest more resources in growth, resulting in increased aboveground plant size (Mahmood et al., 2014). This is partially confirmed in this study as the soils exhibited higher NO_3 and PO_4 contents with increasing age, albeit not significant. Yet, the effects of sterilization in trees grown in 10-year-old forest soils differ from those observed in older forest soils, suggesting that the influence of removing the soil microbiome is contingent on the microbiome of each forest age and cannot be explained entirely

based on higher nutrient availability.

A. glutinosa forms a symbiotic association with the N-fixing bacterium *Frankia alni* which forms nodules on the tree roots and can supply up to 70-90% of the N required by the host tree via fixation of atmospheric N. Thus, we consider that tree reliance on *F. alni* is reflected by the presence and abundance of root nodules (Nickel et al., 2001; Levy-Varon et al., 2019). In the live soils, nodule density significantly decreased with increasing forest age, irrespective of initial *F. alni* abundance in the forest soils. This decrease in nodule density coincided with a decrease in leaf N percentage, an increase in belowground biomass and higher values of root:shoot ratio. This suggests that *F. alni* provided the trees with N and that trees probably invested less in developing their root system to scavenge for N when root nodule density is high (Nickel et al., 2001; Orfanoudakis et al., 2010; Bélanger et al., 2011). Furthermore, trees growing in live 25-year-old soils had a significantly lower percentage of leaf N in comparison to the sterilized soil, while such difference was not present in the younger age classes, possibly because in those younger soils the higher density of root nodules produced by *F. alni* compensated for the lower N availability in the live soil with regards to sterilized forest soil. Although the decrease in leaf N in trees growing in 25-year-old live soils coincides with a decrease in nodule density, nodule density was not correlated with the initial abundance of *F. alni* in the soil. AMFs and EMFs have been shown in the past to positively affect *F.alni* nodulation (Orfanoudakis et al., 2004). On the other hand, AMFs and EMFs are likely to be more abundant as forests age and become more fungal dominated (Kang et al., 2018). Surprisingly, in our study, the relative abundance of EMFs did not increase with increasing forest age and thus it is unlikely to be related to the nodulation decrease in the older treatments of our experiments. One limitation that arises in our study is that the primers used in our analysis do not target AMFs adequately and thus we cannot confidently conclude that AMFs were more abundant with increasing age. Additionally, there was no difference in the relative abundance of pathogenic fungi in the roots of trees between the three age groups and fungi of the *Endogonomycetes* class were not detected at all in the roots. This suggests that other biotic characteristics contributed to reducing root nodulation in trees growing in 25-year-old live soils, and not the abundance of *F. alni* in each of the three forest age groups. Although it is not significant, it could also mean that the increase of available nitrate with age in our soils resulted in the decrease of nodulation as has been shown in the past (Ballhorn et al., 2017). This decrease of nodules in the presence of more available nitrate could also explain the

higher above- and belowground biomass production in 15 and 25yo live soils as trees could instead invest in their roots to scavenge for the readily available nutrients without inhibiting aboveground biomass production due to the (initially) costly symbiosis with *Frankia*.

We observed no significant differences in root-associated bacterial or fungal community composition of trees grown on soil from the different forest ages, despite the different microbial communities present in the initial forest soils, suggesting that *A. glutinosa* selects specific microbiomes. However, we identified key taxa that characterize the soil microbial communities of each forest age. Moreover, there were significant correlations between four key bacterial taxa found in the roots of trees grown in 15-year-old forest soils and aboveground biomass or specific root length, suggesting that, despite the full soil microbial communities not reflecting differences in afforestation stages, there might be crucial players that contribute to increased tree performance along afforestation. Among these, *Actinobacteria* (*Streptomyces* sp.) and *Rhizobiales* that were found in our study, are known to be plant growth-promoting bacteria that are commonly found associated with trees, including alder (Preem et al., 2012; Franco-Correa and Chavarro-Anzola, 2016; Ranjani et al., 2016). The fungal orders *Capnodiales* and *Sordariales,* as well as the family *Aspergillaceae,* contain molds and other species found in decaying plant material of various plant species (Crous et al., 2009; Kendrick, 2017). It is therefore plausible that their presence may have contributed to the reduced root and shoot biomass recorded in trees grown in soil inoculated with forest soil from different ages in comparison to trees growing in just sterilized bulk soil. However, it is improbable that these fungal taxa played a significant role in generating differences among the trees grown in soil inoculated with soil from different forest ages as none of them were significantly negatively correlated with root or shoot biomass. Following that there were also no differences in the relative abundance of fungal pathogens and EMFs in the roots of trees growing in the three ages. We speculate that the role of fungi may be less important than that of bacteria in these early afforestation stages.

There was no effect of age on the microbial community composition in either of the experiments with the exception of the fungal community in experiment 2. However, when evaluating the effects of specific key taxa on plant performance through biotic characteristics alone (experiment 2) compared to biotic and abiotic characteristics combined (experiment 1), the microbial taxa identified to be key players per forest

age varied between the two experiments. This could be attributed to the dilution of the inoculum in the inoculation treatment of experiment 2, as only 10% of live soil was incorporated into bulk soil in each pot. This observation underscores the importance of quantity of inoculum (Han et al., 2022) in determining the communities that are associated with the tree roots. Alternatively, the differences observed between the two experiments could be due to the influence of the abiotic characteristics in the live soils, which were different from those of the bulk sterilized soil, which would lead to selection of the microbes adapted to these particular abiotic conditions, thus acting as a limiting factor for the establishment of particular fungal and bacterial root associated community (Preem et al., 2012).

Many studies have investigated how belowground effects can influence higher trophic levels in plants. These studies have proven that soil microorganisms (eg; bacteria and fungi) and the composition of the soil community can influence plant primary and secondary metabolite concentration (Bezemer et al., 2005) making them more susceptible to herbivory, and also that soil microbial mutualists can improve the nutritional quality of the leaves making them more attractive to herbivores (Ballhorn et al., 2017). In our study, in experiment 1, we observed a decrease in the percentage of N in the leaves of trees growing in live soils with increased forest age which was reflected in decreased herbivory, as expected and previously reported (Ballhorn et al., 2017) and in line with our second hypothesis. Surprisingly, in experiment 2, the percentage of leaf N and herbivory were not significantly associated when assessing the effect of biotic characteristics only. The comparison of the results of the two experiments suggests that biotic characteristics alone may exert specific influences on leaf nutritional characteristics, altering their attractiveness to herbivores, evidently due to N fixing bacteria (Nickel et al., 2001), but likely not exclusively owing to them. This seems to occur independently of leaf N levels, which are typically regarded as a primary determinant of herbivore performance (Whitfeld et al., 2012). Furthermore, it appears that trees growing in 10-year-old soils exhibited the highest herbivory levels, indicating a potential heightened susceptibility to herbivore damage at this early stage of afforestation, influenced by the soil conditions (Xu et al., 2023). However, we cannot definitely discern that this is the case as these assumptions are mainly based on trends and weak correlations. It is important to acknowledge that *Mamestra brassicae* is a generalist herbivore not specialized to *A. glutinosa,* and specialist species could have responded differently to the soil treatments (Kos et al., 2015). However, a study using the generalist gastropod herbivore *Helix pomatia*

showed that, despite the snail's preference to feed on *Urtica dioica*, they still also fed on numerus other plant species in the absence of its preference (Tluste and Birkhofer, 2023), indicating the ability of generalist herbivores to feed on species that they are not commonly found on.

5. Conclusions

In conclusion, our study reveals non-linear responses in *A. glutinosa* performance in relation to soils with different forest ages, with trees growing in 15-year-old forest soils displaying distinct characteristics with respect to the other ages, potentially driven by their unique root microbiome, as indicated by the presence of differentially more abundant microbial taxa that correlate positively with shoot biomass. While we cannot definitively discern whether the disparities in tree and herbivore performance result from the influence of soil biotic or abiotic characteristics, our findings emphasize the impact of changing soil microbiomes with forest age on both plant performance and herbivory. Future studies should expand the scope to include later (>25yo) as well as earlier (<10yo) stages of afforestation and understand the effect of the soil microbiome on tree performance and herbivory in the early and late stages of afforestation. Lastly, since *A. glutinosa* exhibits a unique role as it is both a pioneer species and a N-fixer, future research should also test the aforementioned effects on non N-fixing trees to gain a better understanding of how changes in soil biotic and abiotic properties affect tree performance.

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CRediT authorship contribution statement

Konstantinos Georgopoulos: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft. **T Martijn Bezemer:** Conceptualization, Data curation, Investigation, Methodology,

Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Lisette Neeft:** Data curation, Methodology, Writing – review & editing. **Ana M Camargo:** Writing – review & editing. **Sten Anslan:** Data curation, Software, Writing – review & editing. **Leho Tedersoo:** Data curation, Software, Writing – review & editing. **Sofia IF Gomes:** Data curation, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing.

Author statements

The author Ana M Camargo would like to declare that the positions and opinions presented in this article are those of the authors alone and are not intended to represent the views/any official position or scientific works of the European Food Safety Authority (EFSA).

Supplementary Information

Tables

Table S1 | Soil type and soil properties of the nine forests. Measurements represent the average of the three subplots per forest. Values shown are mean ± se.

Forest identity	Soil type	Soil moisture (%)	SOM (%)	pH	%gravel (≥2mm)	%sand (0.045 – 2 mm)	%silt+clay (<0.045 mm)
1	Podzolic loamy sand	29.98±2.83	11.43±2.59	4.27±0.17	0.00±0.00	96.57±0.59	3.02±0.61
2	Podzolic loamy sand	27.71±1.05	11.61±0.96	4.30±0.08	0.01±0.01	95.83±0.66	3.75±0.65
3	Podzolic loamy sand	17.61±0.76	5.07±0.36	5.33±0.14	0.03±0.03	91.15±0.58	8.13±0.55
4	Podzolic loamy sand	42.08±8.48	18.22±5.19	4.07±0.05	0.03±0.02	96.56±0.07	2.89±0.07
5	Podzolic loamy sand	18.41±1.24	5.67±0.68	4.19±0.06	0.00±0.00	97.80±0.15	1.81±0.21
6	Podzolic loamy sand	17.37±1.90	5.05±0.13	4.52±0.03	0.00±0.00	96.14±0.26	3.64±0.22
7	Podzolic loamy sand	23.82±1.94	6.70±0.62	5.60±0.08	0.18±0.18	85.00±2.40	14.05±2.16
8	Podzolic loamy sand	27.05±2.28	6.86±0.48	5.39±0.26	0.02±0.02	88.40±3.64	10.77±3.47
9	Podzolic loamy sand	27.78±1.07	10.09±0.14	5.70±0.26	0.00±0.00	94.33±0.53	4.96±0.45
Control	Bulk sterilized	-	7.59±0.29	8.18±0.04	0.08	98.11	1.40

Table S2 | General information of each forest from the chronosequence.

Forest	Year of afforestation	Former Land use	Latitude	Longitude	Province	Surface (ha)
1	1994	Cropland	52°59'51.33"N	6°53'28.20"E	Drenthe	221.1
2	1996	Cropland	53°7'17.15"N	6°52'0.91"E	Groningen	19.8
3	1996	Cropland/ Pasture	53°3'31.17"N	6°40'44.62"E	Drenthe	124.8
4	2005	Cropland	52°41'58.61"N	6°49'14.59"E	Drenthe	6.15
5	2005	Cropland	52°53'15.98"N	6°24'32.26"E	Drenthe	7.33
6	2005	Cropland	52°47'14.89"N	6°35'56.38"E	Drenthe	19.63
7	2011	Cropland/ Pasture	53°2'36.25"N	6°31'52.62"E	Drenthe	4.58
8	2010	Cropland	53°2'28.09"N	6°32'6.39"E	Drenthe	-
9	2012	Cropland	52°59'57.04"N	6°52'58.37"E	Drenthe	30.03

Table S3 | Soil ammonium (NH_4), nitrate (NO_3) and orthophosphate (PO_4) of the nine forests. Measurements represent the average of the three subplots per forest. Values shown are mean ± se.

Forest	NH_4 (mg/kg soil)	NO_3 (mg/kg soil)	PO_4 (mg/kg soil)
1	80.56±0.39	37.54±1.01	3.54±1.29
2	402.41±215.05	22.95±0.59	2.62±1.14
3	40.96±4.37	31.91±2.99	0.85±0.84
4	372.38±273.93	25.21±0.89	3.47±1.13
5	117.09±19.60	25.15±2.63	1.15±0.95
6	40.69±18.76	26.06±0.80	0.99±0.98
7	83.23±11.09	16.79±0.62	0.43±1.28
8	39.18±20.91	34.56±0.04	0.82±1.14
9	106.25±53.55	19.04±0.37	1.03±0.85

Table S4 | Results from a linear mixed effects model (LMM) testing the effects of forest Age, Treatment and their interaction on A. glutinosa Aboveground biomass, Belowground biomass, Percentage fine roots, Root/Shoot ratio, Nodule density, Stem width, Specific root length (SRL) and Percentage leaf N. Presented in the table are the degrees of freedom (DF), the $\chi 2$ and the p-value (p). In cases of significant results ($p < 0.05$), the p-values are bolded.

Effect	DF	χ^2	p
Aboveground biomass			
Age	2	3.732	0.154
Treatment	1	11.987	0.835
Age:Treatment	2	5.423	0.066
Belowground biomass			
Age	2	3.586	0.166
Treatment	1	0.915	0.847
Age:Treatment	2	0.703	0.703
Percentage fine roots			
Age	2	0.506	0.776
Treatment	1	18.773	0.608
Age:Treatment	2	7.534	**0.023**
Root/Shoot ratio			
Age	2	0.544	0.761
Treatment	1	3.011	**0.032**
Age:Treatment	2	0.080	0.960
Nodule density			
Age	2	5.461	**<0.001**
Treatment	1	3.265	**<0.001**
Age:Treatment	2	10.251	**0.031**
Stem width			
Age	2	0.441	0.801
Treatment	1	2.117	0.192
Age:Treatment	2	7.311	**0.025**
SRL			
Age	2	0.078	0.827
Treatment	1	9.715	0.228
Age:Treatment	2	7.581	**0.038**
Percentage leaf N			
Age	2	3.957	0.138
Treatment	1	4.272	**0.038**
Age:Treatment	2	1.781	0.410

Table S5 | Results from the linear mixed effects model (LMM) which tests the effects of forest age on A. glutinosa Aboveground biomass, Belowground biomass, Percentage fine roots, Root/Shoot ratio, Nodule density, Stem width, Specific root length (SRL) and Percentage leaf N based on a LMM including the control treatment. Presented in the table are the degrees of freedom (DF), the $\chi 2$ and the p-value (p).

Effect of Age	DF	χ^2	p
Aboveground biomass	3	6.342	0.096
Belowground biomass	3	10.87	**0.012**
Percentage Fine roots	3	4.081	0.252
Root/Shoot ratio	3	13.642	**0.003**
Nodule density	3	9.136	**0.03**
Stem width	3	10.099	**0.018**
SRL	3	6.247	0.100
Percentage leaf N	3	8.601	**0.035**

Table S6 | Results from the linear mixed effects model (LMM) which excludes the control treatment to test the effects of forest age on A. glutinosa Aboveground biomass, Belowground biomass, Percentage fine roots, Root/Shoot ratio, Nodule density, Stem width, Specific root length (SRL) and Percentage leaf N. Presented in the table are the degrees of freedom (DF), the $\chi 2$ and the p-value (p).

Effect of Age	DF	χ^2	p
Aboveground biomass	2	6.275	**0.043**
Belowground biomass	2	3.172	0.204
Percentage fine roots	2	3.791	0.150
Root/Shoot ratio	2	2.138	0.343
Nodule density	2	1.752	0.416
Stem width	2	3.029	0.219
SRL	2	5.070	0.079
Percentage leaf N	2	4.232	0.120

Table S7 | Classification of bacterial ASVs with differentially abundant taxa in experiment 1 down to Genus level. Colors indicate the age where those taxa were found to be differentially abundant (yellow = 10yo, blue = 15yo and green = 25yo)

ASV	Phylum	Class	Order	Family	Genus
33	Proteobacteria	Gammaproteobacteria	Burkholderiales	*Hydrogenophilaceae*	*Tepidiphilus*
47	Actinobacteriota	Actinobacteria	Micrococcales	*Microbacteriaceae*	*Galbitalea*
108	Methylomirabilota	Methylomirabilia	Rokubacteriales		
122	Proteobacteria	Gammaproteobacteria	Burkholderiales	*Burkholderiaceae*	*Burkholderia-Caballero-nia-Paraburkholderia*
131	Proteobacteria	Gammaproteobacteria	Burkholderiales	*Rhodocyclaceae*	*Methyloversatilis*
132	Proteobacteria	Alphaproteobacteria	Rhizobiales	*Rhizobiaceae*	*Neorhizobium*
140	Proteobacteria	Alphaproteobacteria	Rhizobiales	*Xanthobacteraceae*	
142	Proteobacteria	Alphaproteobacteria	Rhodobacterales	*Rhodobacteraceae*	
148	Proteobacteria	Gammaproteobacteria	Gammaproteobacteria Incertae Sedis	*Unknown Family*	*Acidibacter*
150	Proteobacteria	Alphaproteobacteria	Caulobacterales	*Caulobacteraceae*	*Asticcacaulis*
152	Actinobacteriota	Actinobacteria	Frankiales	*Acidothermaceae*	*Acidothermus*
158	Actinobacteriota	Actinobacteria	Streptomycetales	*Streptomycetaceae*	*Streptomyces*
182	Planctomycetota	Planctomycetes	Pirellulales	*Pirellulaceae*	
189	Proteobacteria	Alphaproteobacteria	Rhizobiales	*Devosiaceae*	*Devosia*
197	Proteobacteria	Gammaproteobacteria	Burkholderiales	*A21b*	
198	Proteobacteria	Alphaproteobacteria	Elsterales		
204	Proteobacteria	Gammaproteobacteria	Methylococcales	*Methylococcaceae*	*Methylocaldum*
220	Proteobacteria	Alphaproteobacteria	Rhizobiales	*Xanthobacteraceae*	
238	Proteobacteria	Alphaproteobacteria	Rhizobiales	*Methyloligellaceae*	
250	Proteobacteria	Gammaproteobacteria	Burkholderiales	*Burkholderiaceae*	*Burkholderia-Caballero-nia-Paraburkholderia*
268	Actinobacteriota	Actinobacteria	Streptomycetales	*Streptomycetaceae*	*Streptomyces*
278	Nitrospirota	Nitrospiria	Nitrospirales	*Nitrospiraceae*	*Nitrospira*
279	Methylomirabilota	Methylomirabilia	Rokubacteriales		
280	Proteobacteria	Gammaproteobacteria	Burkholderiales	*Methylophilaceae*	*Methylophilus*
287	Cyanobacteria	Cyanobacteriia	Cyanobacteriales	*Nostocaceae*	*Cylindrospermum PCC-7417*
300	Proteobacteria	Alphaproteobacteria	Acetobacterales	*Acetobacteraceae*	
301	Proteobacteria	Alphaproteobacteria	Micropepsales	*Micropepsaceae*	
306	Actinobacteriota	Acidimicrobiia	Microtrichales		
334	Proteobacteria	Alphaproteobacteria	Acetobacterales	*Acetobacteraceae*	
351	Planctomycetota	Planctomycetes	Pirellulales	*Pirellulaceae*	*Pir4 lineage*
368	Actinobacteriota	Actinobacteria	Frankiales	*Acidothermaceae*	*Acidothermus*

393	Proteobacteria	Gammaproteobacteria	Xanthomonadales	*Rhodanobacteraceae*	*Rhodanobacter*
397	Proteobacteria	Alphaproteobacteria	Sphingomonadales	*Sphingomonadaceae*	*Sphingomonas*
400	Proteobacteria	Alphaproteobacteria			
405	Planctomycetota	Planctomycetes	Isosphaerales	*Isosphaeraceae*	
417	Proteobacteria	Alphaproteobacteria	Rhizobiales	*Beijerinckiaceae*	*Roseiarcus*
420	Proteobacteria	Alphaproteobacteria	Rhizobiales	*Devosiaceae*	
421	Proteobacteria	Gammaproteobacteria	Burkholderiales	*SC-I-84*	
455	Proteobacteria	Gammaproteobacteria	Burkholderiales	*Comamonadaceae*	*Rhizobacter*
469	Proteobacteria	Alphaproteobacteria	Rhizobiales	*Methyloligellaceae*	
478	Proteobacteria	Alphaproteobacteria	Rhizobiales	*Xanthobacteraceae*	
485	Proteobacteria	Gammaproteobacteria	Xanthomonadales	*Rhodanobacteraceae*	
507	Proteobacteria	Alphaproteobacteria	Rhizobiales	*Xanthobacteraceae*	*Pseudolabrys*
523	Actinobacteriota	Actinobacteria	Frankiales	*Acidothermaceae*	*Acidothermus*
535	Proteobacteria	Alphaproteobacteria	Micropepsales	*Micropepsaceae*	
541	Proteobacteria	Alphaproteobacteria	Rhizobiales	*Rhizobiaceae*	
546	Proteobacteria	Alphaproteobacteria	Rhizobiales	*Hyphomicrobiaceae*	
571	Proteobacteria	Alphaproteobacteria	Elsterales		
572	Actinobacteriota	Actinobacteria	Streptomycetales	*Streptomycetaceae*	*Streptomyces*
573	Proteobacteria	Alphaproteobacteria	Rhizobiales	*Rhizobiaceae*	
585	Actinobacteriota	Actinobacteria	Micromonosporales	*Micromonosporaceae*	*Actinoplanes*
594	Proteobacteria	Alphaproteobacteria	Rhodobacterales	*Rhodobacteraceae*	*Paracoccus*
626	Proteobacteria	Gammaproteobacteria	Xanthomonadales	*Rhodanobacteraceae*	*Dyella*
637	Proteobacteria	Alphaproteobacteria	Micropepsales	*Micropepsaceae*	
639	Proteobacteria	Alphaproteobacteria	Rhizobiales	*Xanthobacteraceae*	*Pseudolabrys*
648	Planctomycetota	Planctomycetes	Planctomycetales		
659	Proteobacteria	Alphaproteobacteria	Caulobacterales	*Caulobacteraceae*	
666	Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Moraxellaceae*	*Acinetobacter*
671	Proteobacteria	Alphaproteobacteria	Rhizobiales	*Beijerinckiaceae*	*Methylocapsa*
676	Proteobacteria	Alphaproteobacteria	Caulobacterales	*Caulobacteraceae*	
691	Proteobacteria	Alphaproteobacteria	Rhizobiales	*Rhizobiaceae*	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*
715	Proteobacteria	Gammaproteobacteria	Burkholderiales	*Comamonadaceae*	
718	Actinobacteriota	Actinobacteria	Micrococcales	*Cellulomonadaceae*	*Actinotalea*
720	Actinobacteriota	Actinobacteria	Corynebacteriales	*Mycobacteriaceae*	*Mycobacterium*
745	Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Pseudomonadaceae*	*Pseudomonas*

780	Actinobacteriota	Actinobacteria	Streptosporangiales	*Streptosporangiaceae*	*Streptosporangium*
854	Proteobacteria	Gammaproteobacteria	Burkholderiales	*Comamonadaceae*	
880	Proteobacteria	Gammaproteobacteria	JG36-TzT-191		
890	Proteobacteria	Alphaproteobacteria	Elsterales		
923	Proteobacteria	Gammaproteobacteria	Gammaproteobacteria Incertae Sedis	*Unknown Family*	*Acidibacter*
951	Proteobacteria	Alphaproteobacteria	Reyranellales	*Reyranellaceae*	*Reyranella*
954	Myxococcota	Polyangia	Polyangiales	*Sandaracinaceae*	*Sandaracinus*
963	Proteobacteria	Gammaproteobacteria	Burkholderiales	*Comamonadaceae*	*Hydrogenophaga*
996	Proteobacteria	Gammaproteobacteria	Burkholderiales	*Burkholderiaceae*	*Burkholderia-Caballeronia-Paraburkholderia*
1072	Proteobacteria	Alphaproteobacteria	Micropepsales	*Micropepsaceae*	
1083	Proteobacteria	Gammaproteobacteria	Diplorickettsiales	*Diplorickettsiaceae*	
1085	Proteobacteria	Gammaproteobacteria	Xanthomonadales	*Rhodanobacteraceae*	*Dokdonella*
1086	Bacteroidota	Bacteroidia	Chitinophagales	*Chitinophagaceae*	*Puia*
1127	Proteobacteria	Alphaproteobacteria	Acetobacterales	*Acetobacteraceae*	
1145	Actinobacteriota	Actinobacteria	Frankiales	*Acidothermaceae*	*Acidothermus*
1153	Proteobacteria	Alphaproteobacteria	Micropepsales	*Micropepsaceae*	
1157	Actinobacteriota	Actinobacteria	Propionibacteriales	*Propionibacteriaceae*	*Cutibacterium*
1198	Proteobacteria	Alphaproteobacteria	Acetobacterales	*Acetobacteraceae*	
1211	Proteobacteria	Alphaproteobacteria	Caulobacterales	*Caulobacteraceae*	
1241	Proteobacteria	Alphaproteobacteria	Rhizobiales	*Devosiaceae*	
1259	Actinobacteriota	Actinobacteria	Streptomycetales	*Streptomycetaceae*	
1279	Proteobacteria	Gammaproteobacteria	Burkholderiales	*Comamonadaceae*	*Ramlibacter*
1292	Proteobacteria	Alphaproteobacteria	Rhizobiales	*Xanthobacteraceae*	
1371	Myxococcota	Polyangia	Polyangiales	*Polyangiaceae*	*Pajaroellobacter*
1783	Proteobacteria	Gammaproteobacteria	Steroidobacterales	*Steroidobacteraceae*	*Steroidobacter*
2871	Proteobacteria	Alphaproteobacteria	Elsterales		

Table S8 | Classification of fungal OTUs with differentially abundant taxa in experiment 1 down to Genus level. Colors indicate the age where those taxa were found to be differentially abundant (yellow = 10yo, blue = 15yo and green = 25yo)

OTU	Phylum	Class	Order	Family	Genus
53	Ascomycota	Dothideomycetes	Pleosporales	*Dictyosporiaceae*	*Pseudocoleophoma*
206	Ascomycota	Dothideomycetes	Pleosporales	*Phaeosphaeriaceae*	*Phaeosphaeria*
242	Ascomycota	Dothideomycetes	Pleosporales	*Dothidotthiaceae*	*Pleiochaeta*

Table S9 | Correlations between abundant taxa and measured variables in experiment 1.

ASV	Taxa	R^2	p	Correlated variable
ASV158	*Streptomyces* sp.	0.22	**0.010**	**Aboveground biomass**
ASV279	*Rokubacteriales*	0.20	**0.010**	**Aboveground biomass**
ASV421	*Burkhoderiales*	0.26	**0.010**	SRL
ASV306	*Microtrichales*	0.27	**0.010**	SRL
ASV250	*Burkhoderiales*	0.28	**0.010**	SRL
ASV268	*Streptomyces* sp.	0.34	**0.010**	SRL

Table S10 | Classification of fungal OTUs with differentially abundant taxa in experiment 2 down to Genus level. Colors indicate the age where those taxa were found to be differentially abundant (yellow = 10yo, blue = 15yo and green = 25yo)

OTU	Phylum	Class	Order	Family	Genus
48	Ascomycota	Leotiomycetes	Thelebolales	*Pseudeurotiaceae*	
371	Ascomycota	Dothideomycetes	Capnodiales		
1170	Ascomyctoa	Saccharomycetes	Saccharomy-cetales	*Phaffomycetaceae*	*Cyberlindnera*
1387	Rozellomycota				
1591	Ascomycota	Eurotiomycetes	Eurotiales	*Aspergillaceae*	
1681	Ascomycota	Eurotiomycetes	Chaetothyriales	*Herpotrichiella-ceae*	*Cladophialophora*
3056	Rozellomycota				
3072	Ascomycota	Eurotiomycetes	Verrucariales	*Verrucariaceae*	*Polyblastia*
3144	Ascomycota	Orbiliomycetes	Orbiliales	*Orbiliaceace*	*Orbilia*

Figures

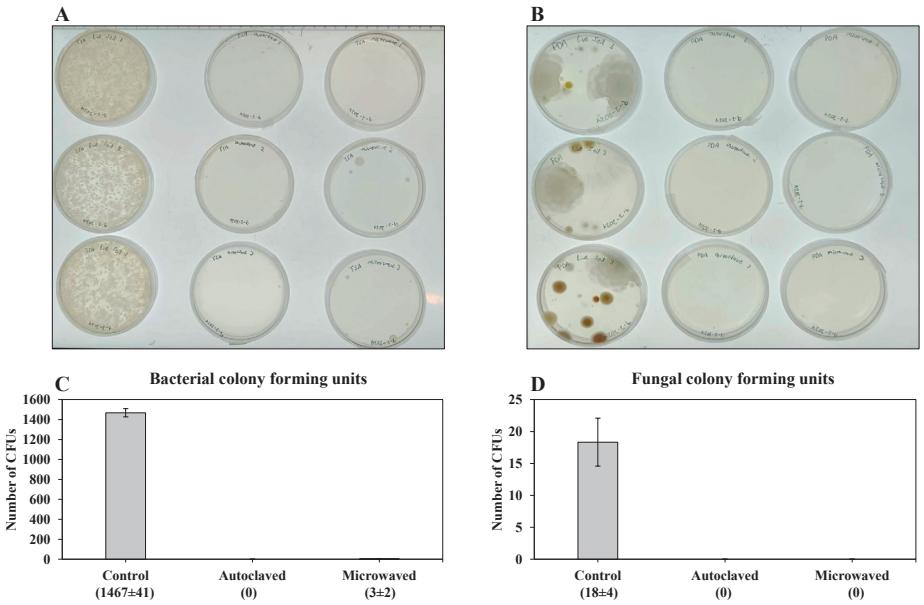

Figure S1 | Comparison of autoclaving and microwaving soil to remove the soil microbiome. Autoclaved and microwaved soil was mixed with sterilized H2O (1:1 ratio) and left to settle for five minutes. Hereafter, 200 µl of the liquid suspension was plated on (A) tryptic soy agar (TSA) and (B) potato dextrose agar (PDA) plates (n = 3) and left in the dark, at room temperature (20 °C) for one week. The same was done using live soil (Control). After three days for bacteria (C) and one week for fungi (D), the colony forming units (CFU) were counted to assess the effectiveness of the sterilization and microwaving methods. Autoclaving the soil lead to the complete absence of CFUs for both bacteria and fungi. Microwaving the soil lead to a 99.8% reduction in bacterial CFUs and a complete absence of fungal CFUs. Thus, for the rest of the document we assume that sterilization led to the removal of the soil microbiome. Presented in the bar plots are the CFU means ± se.

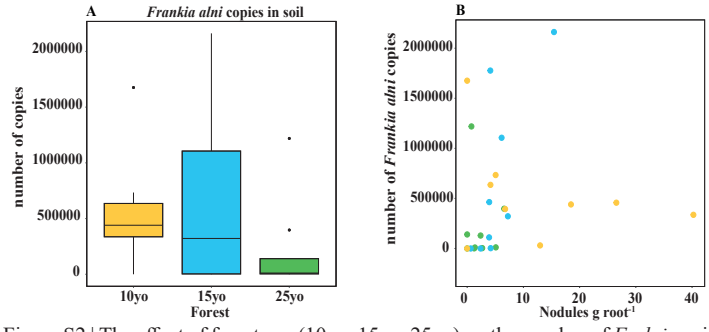

Figure S2 | The effect of forest age (10yo, 15yo, 25yo) on the number of *F. alni* copies found in the chronosequence forest soils from qPCR analysis (A) and the correlation between the Nodule density and the number of *F. alni* copies found in the chronosequence forest soils (B).

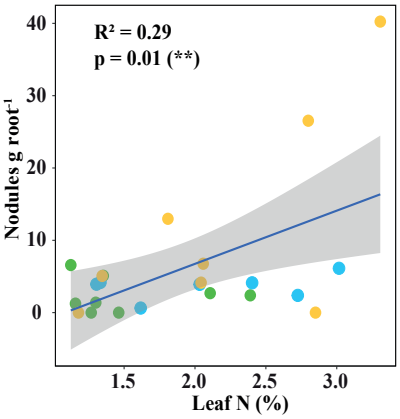

Figure S3 | Correlation between the percentage leaf N and root nodule density in the live mesocosms of experiment 1. Asterisks represent significant p-values. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

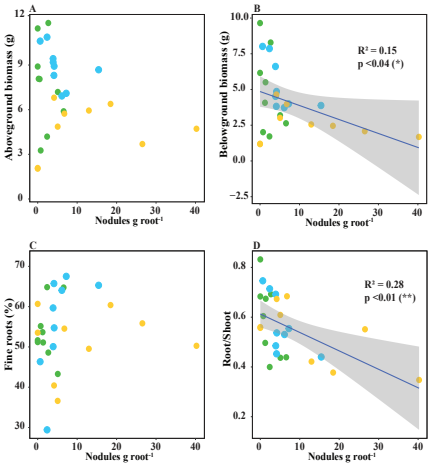

Figure S4 | Correlation between the root nodule density and (A) Aboveground Biomass (B) Belowground Biomass (C) Fine roots (%) and (D) Root/Shoot ratio in experiment 1. Asterisks represent significant p-values. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

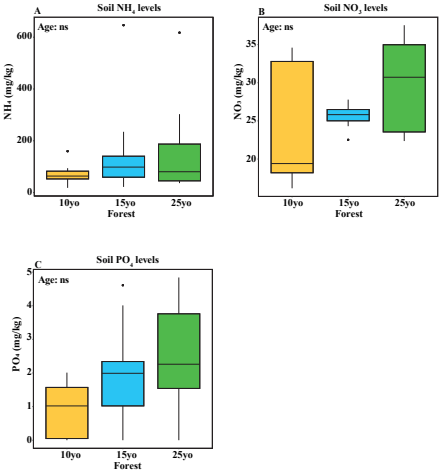

Figure S5 | The effect of forest age (10yo, 15yo, 25yo) on the NH_4 (A), NO3 (B) and PO4 (C) levels measured from the chronosequence forest soils.

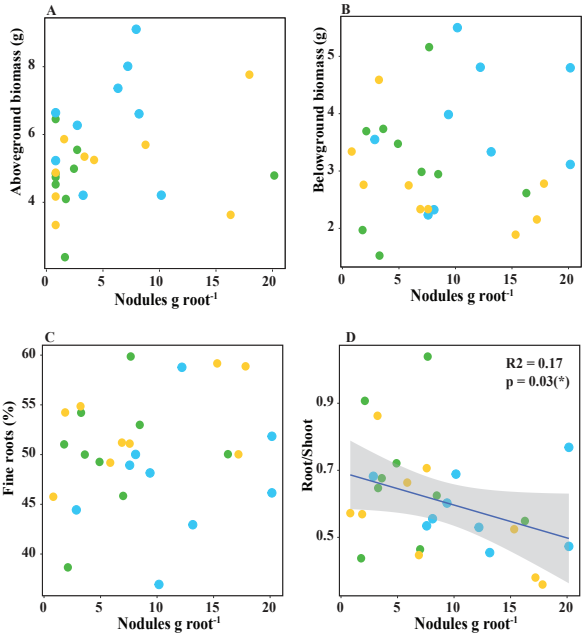

Figure S6 | Correlation between the root nodule density and (A) Aboveground Biomass (B) Belowground Biomass (C) Percentage fine roots and (D) Root/Shoot ratio in experiment 2. Asterisks represent significant p-values. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

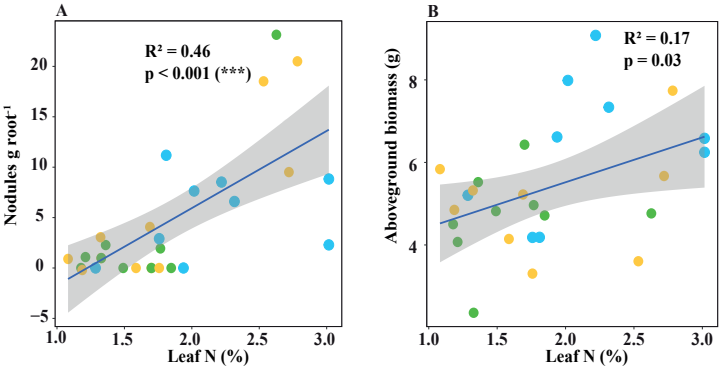

Figure S7 | Correlation between the percentage leaf N (A) Root nodule density and (B) Aboveground biomass in experiment 2. Asterisks represent significant p-values. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

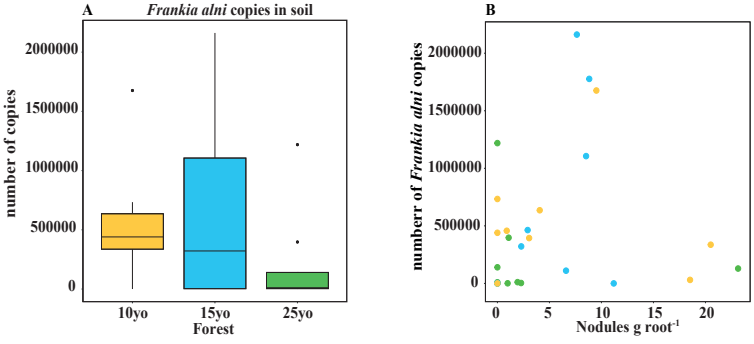

Figure S8 | The effect of forest age (10yo, 15yo, 25yo) on the number of F. alni copies found in the chronosequence forest soils from qPCR analysis (A) and the correlation between the Nodule density and the number of F. alni copies found in the chronosequence forest soils (B).

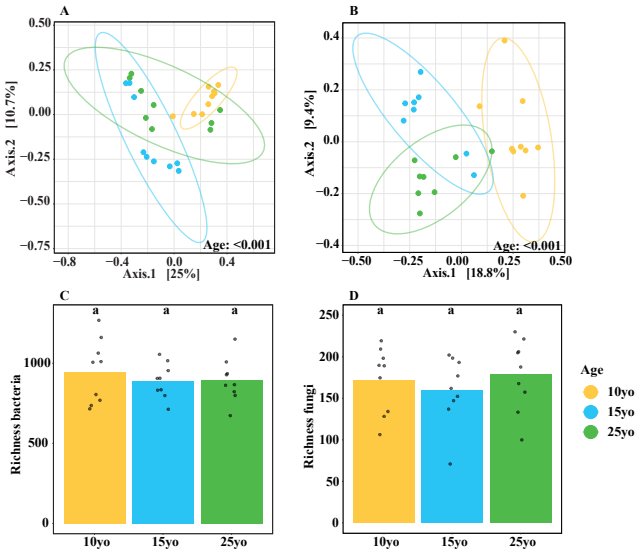

Figure S9 | Soil bacterial (A) and fungal (B) community composition (based on Bray-Curtis similarity), soil bacterial (C) and fungal (D) alpha diversity and the number of shared and unique soil bacterial ASVs (E) and fungal OTUs (F).

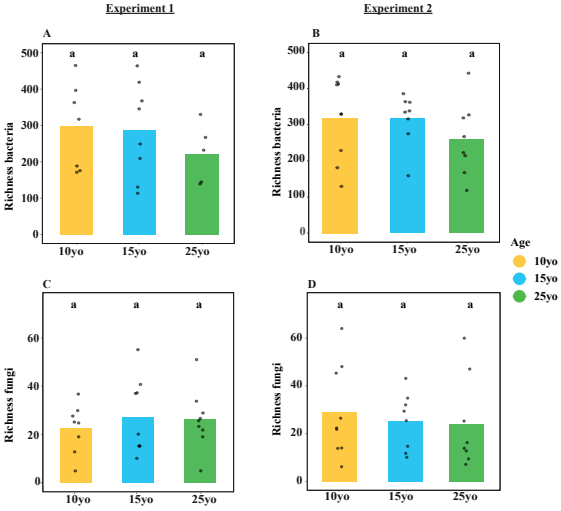

Figure S10 | Root bacterial (A, B) and fungal (C,D) alpha diversity in experiment 1 (left) and experiment 2 (right).

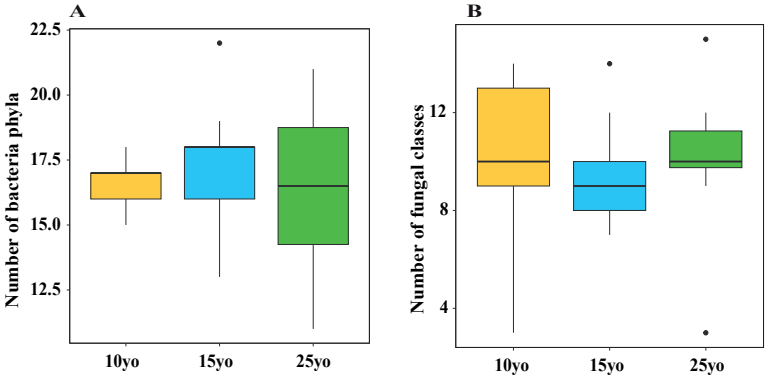

Figure S11 | The number of root associated bacterial phyla (A) and fungal classes (B) from Experiment 1. The error bars represent the 95% confidence interval with the bottom and top of the box being the 25th and 75th percentiles respectively. The line inside the box represents the median.

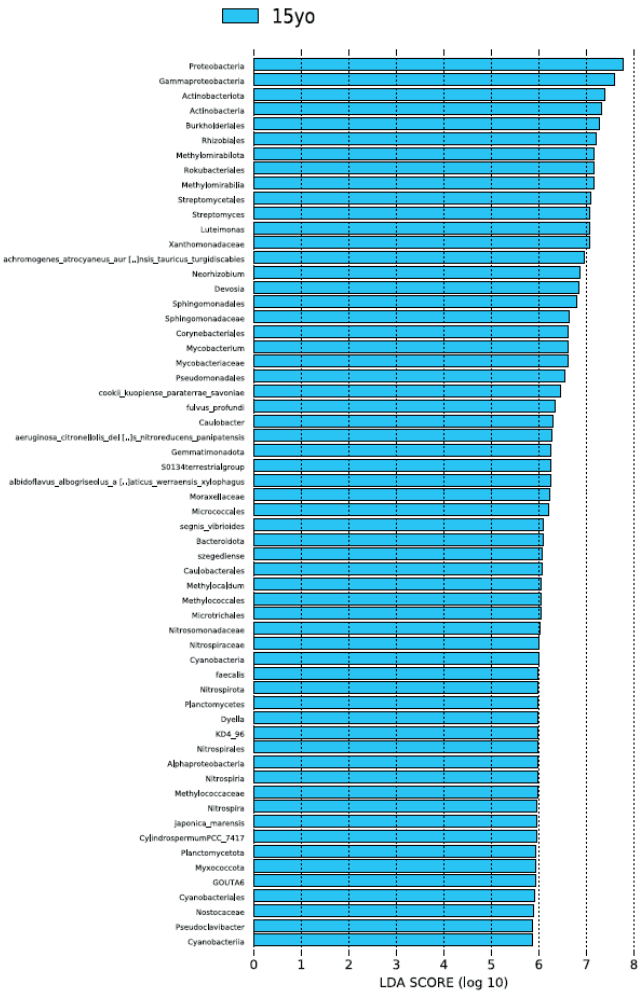

Figure S12 | Output of the LEfSe analysis depicting the root associated bacterial taxa that are differentially abundant between 10, 15 and 25yo forests in Experiment 1. A Kruskal-Wallis sum rank test is used to detect significant differential abundance of bacteria taxa with respect to forest age, after which pairwise tests are performed using a Wilcoxon rank-sum test.

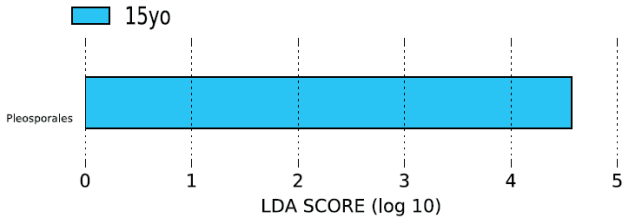

Figure S13 | Output of the LEfSe analysis depicting the root associated fungal taxa that are differentially abundant between 10, 15 and 25yo forests in Experiment 1. A Kruskal-Wallis sum rank test is used to detect significant differential abundance of bacteria taxa with respect to forest age, after which pairwise tests are performed using a Wilcoxon rank-sum test.

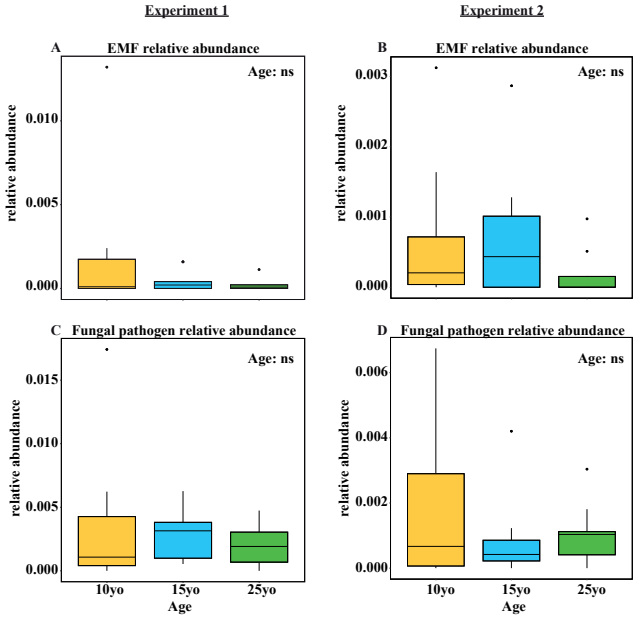

Figure S14 | The effect of age on the relative abundance of EMFs (A,B) and fungal pathogens (C,D) in the roots of trees from experiment 1 (left) and experiment 2 (right).

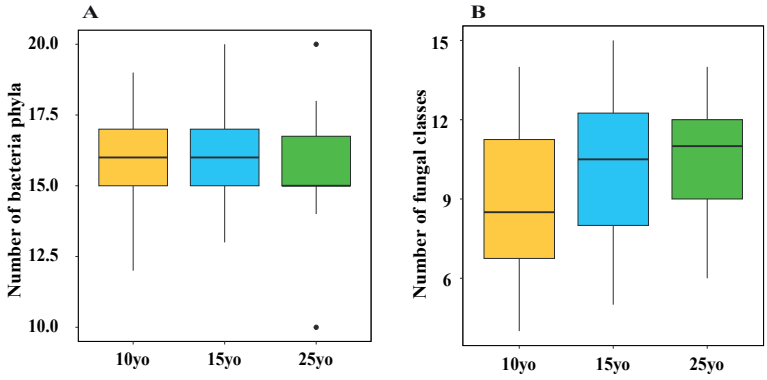

Figure S15 | The number of root associated bacterial phyla (A) and fungal classes (B) from Experiment 2. The error bars represent the 95% confidence interval with the bottom and top of the box being the 25th and 75th percentiles respectively. The line inside the box represents the median.

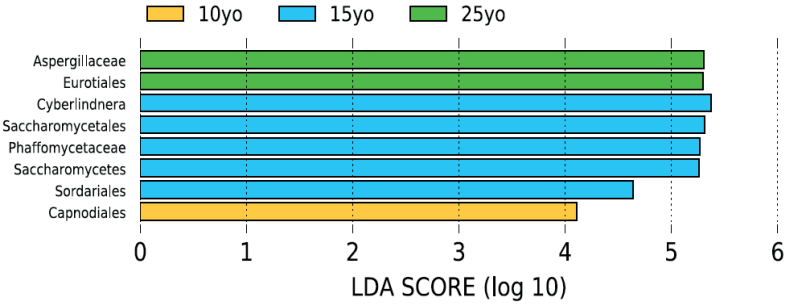

Figure S16 | Output of the LEfSe analysis depicting the root associated fungal taxa that are differentially abundant between 10, 15 and 25yo forests in Experiment 2. A Kruskal-Wallis sum rank test is used to detect significant differential abundance of bacteria taxa with respect to forest age, after which pairwise tests are performed using a Wilcoxon rank-sum test.

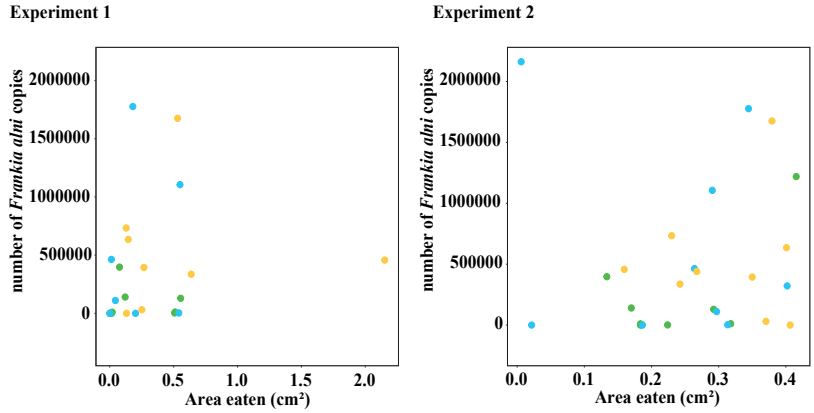

Figure S17 | The correlation between the leaf area eaten by *Mamestra brassicae* and the number of *F. alni* copies found in the chronosequence forest soils from qPCR analysis.