Tree-distance and tree-species effects on soil biota in a temperate agroforestry system

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Abstract

Aims Cropland agroforestry systems are land-use systems with numerous environmental advantages over monoculture croplands including promotion of soil life. This study aimed to investigate tree-species and tree-distance effects on soil biota in a temperate agroforestry system.

Methods Our study was conducted at a paired alley-cropping and monoculture cropland system. The tree rows of the agroforestry system comprised of blocks of poplar Fritzi Pauley, poplar Max 1 or black locust. Within the agroforestry system, soil microbial and earthworm communities were collected along transects spanning from the center of the tree rows into the crop rows. Archaea, bacteria, and fungi were quantified using real-time PCR. The community composition of fungi and earthworms was deciphered using amplicon sequencing and morphological identification, respectively.

Results Tree rows promoted the abundance of bacteria and earthworms, which we attribute mainly to tree litter input and the absence of tillage. Fungal community composition was altered by the tree rows, resulting in an increased proportion of ectomycorrhizal fungi in the tree-row associated mycobiome. The proportion of *Blumeria graminis,* the causal agent of powdery mildew, increased with increasing distance from the trees. We suggest that enhanced microbial antagonism, increased earthworm densities and/or altered microclimate contributed to the suppression of *B. graminis* in vicinity of the trees. Tree-species effect had a minor influence on the abundance and composition of soil communities at our study site.

Conclusions In comparison to monoculture cropland, agroforestry benefits the abundance, diversity, and function of soil biota and may enhance soil suppressiveness.

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Introduction

Agroforestry systems are land-use systems that combine trees with crops and/or livestock. In the temperate zone, alley-cropping agroforestry systems that alternate rows of trees with rows of crops are gaining popularity. Tree rows of these systems usually consist of either fast-growing trees (e.g. poplar or willow) for biomass production or quality hardwoods such as cherry or walnut trees. The environmental benefits of temperate agroforestry practice are well recognized and include, *inter alia*, increased carbon sequestration (e.g. Mayer et al. 2022), reduced nutrient leaching (e.g. Allen et al. 2004; Wang et al. 2011), reduced soil erosion, and promotion of biodiversity (e.g. Varah et al. 2013, 2020). Certain advantages of alley-cropping systems over monoculture croplands, such as the complementary use of resources, are due to interspecific interactions between the trees and the crops (Jose et al. 2004). For example, deep-rooting trees are able to take up nutrients leached below the rooting zone of the crops ('safety-net'-function of the trees; Allen et al. 2004) and make them available to crops via litter fall. This process was recently dubbed as 'nutrient pumping' (Isaac and Borden 2019) and is supported by findings of increased soil nutrient availability in vicinity of the tree rows of temperate agroforestry systems (Pardon et al. 2017).

Soil biota are known to predominantly benefit from temperate agroforestry practice (Beule et al. 2022a; Marsden et al. 2020). Several studies on soil microorganisms in alley-cropping systems revealed that the tree rows promote microbial population size and that this effect is not only limited to the tree rows but extends gradually into the crop rows (i.e. stronger promotion in vicinity of the trees) (e.g. Beule et al. 2022b; Guillot et al. 2021). Furthermore, soil microbiome studies reported that the composition of bacterial and fungal communities strongly differs between the tree and crop rows (e.g. Beule et al. 2021; Beule & Karlovsky 2021a). Based on these studies, it was recently reviewed that alley-cropping systems increase microbial diversity in soil through increased beta diversity rather than alpha diversity (Beule et al. 2022a). Recent articles showed that soil fungal communities strongly respond to temperate agroforestry practices. For example, one study found that Basidiomycota in soil were up to 330 times more abundant in poplar tree rows of alley-cropping systems as

compared to adjacent cropland monocultures (Beule et al. 2021). The same study used amplicon sequencing to investigate the composition of the soil fungal community and found that certain ectomycorrhizal fungi (*Cortinarius*, *Geopora*, and *Inocybe*) were particularly promoted by poplar trees, which holds great potential to improve nutrient acquisition in agroforestry systems (Beule et al. 2021). In the present study, we aimed to explore the impact of different tree species on soil fungal communities in agroforestry systems.

In 1999, Seiter and co-workers (1999) observed that most of the tree leaf litter in an alder–maize alley-cropping system was pulled into the burrows by *Lumbricus terrestris* but did not provide data to substantiate their field observation. Few months earlier, Price and Gordon (1998) published an article demonstrating tree-species specific promotion of earthworms through tree rows in an alley-cropping system. Since then, greater densities of earthworms in tree rows of temperate agroforestry systems as compared to bordering crop rows or adjacent monoculture croplands have been reported (Cardinael et al. 2019; D'Hervilly et al. 2022). Yet, studies investigating earthworm communities in temperate agroforestry systems along fine spatial gradients from the tree rows into the crop rows comprising more than two distances from the tree rows are scarce. Furthermore, most temperate agroforestry systems feature either a single tree species or an intermixture of tree species (e.g. Cardinael et al. 2019), thus disabling direct comparisons among tree species within the same system. To our best knowledge, the investigation by Price and Gordon (1998) is the only study to assess earthworm communities under different tree species within the same temperate agroforestry system. However, the authors did not report species identities or ecological groups of collected earthworms. Recent studies indicated differences in the response of different ecological groups of earthworms to agroforestry practice (Cardinael et al. 2019; D'Hervilly et al. 2022), highlighting the importance of investigating them.

In the present study, we chose two poplar species, as poplar trees are the most commonly planted short-rotation trees in modern alley-cropping agroforestry systems in the temperate zone. Although not commonly planted in temperate agroforestry systems, we further chose black locust as it is a fast-growing, nitrogen-fixing tree species. We expect differences

in overall soil biota communities under different tree species because root architecture, litter quality, and soil-nutrient cycling are tree species-specific (e.g. Das and Chaturvedi 2008, Aponte et al. 2013, Borden et al. 2017).

This study aimed to investigate the impact of three different tree species (two poplar species and black locust) on representatives of the soil biota community (archaea, bacteria, fungi, and earthworms) in a temperate alley-cropping agroforestry system. Furthermore, tree-distance effects on soil biota were tested by sampling multiple locations along transects spanning from the tree row into the crop row of the agroforestry system. We hypothesized that tree rows promote the abundance and alter the community composition of soil biota. We further hypothesized that these changes are dependent on the distance to the trees (tree-distance effect) as well as the tree species (tree-species effect).

Materials and Methods

Study site and sampling design

Our study site was located on a Gleyic Cambisol soil (IUSS Working Group WRB 2015) near Forst, Brandenburg, Germany (51°47′11″N, 14°38′05″E; m.a.m.s.l.: 67 m; mean annual temperature: 9.6 ± 0.2 °C; mean annual precipitation: 568 ± 21 mm; Fig. 1A), which is located in the glacially influenced region of the North German Plain. General biochemical and physical soil properties of the site were recently characterized by Schmidt et al. (2021). According to aerial images, the study site was under agricultural use for at least 50 years prior to conversion of cropland monoculture to alley-cropping agroforestry. At the study site, a conventionally managed alley-cropping agroforestry system was spatially paired with a conventional monoculture cropland system that served as a reference land use. The agroforestry system was established in 2010 and was 12 years old during sampling. The system consisted of 12 m-wide rows of trees (north–south orientation) that were alternated with 48 m-wide rows of arable crops (Fig. 1B). Tree rows were hand planted and consisted of three different tree species. The tree rows comprised four individual rows of trees (Fig. 1 C, D, E). The tree rows that defined the crop row consisted of three different tree species that were planted in alternating segments at a length of approx. 165 m (Fig. 1B). The different tree species were i) *Populus trichocarpa* cv. Fritzi Pauley (referred to as 'poplar Fritzi Pauley'; Fig. 1C), ii) *P. nigra × P. maximowiczii*
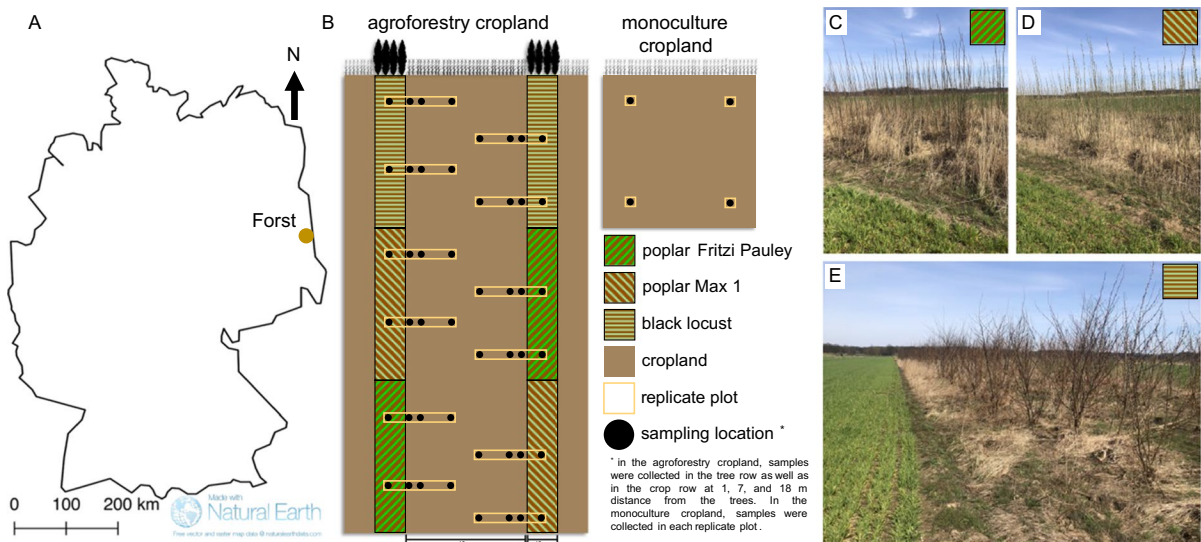

Fig. 1 Study site and study design. Location of the study site near Forst (federal state of Brandenburg), Germany (**A**), study design (**B**), and photos of the three different tree species cultivated at the site (poplar Fritzi Pauley (**C**), poplar Max 1 (**D**), and black locust (**E**)) taken in April 2022. Photo credit: Lukas Beule

cv. Max 1 (referred to as 'poplar Max 1'; Fig. 1D), and iii) black locust (*Robinia pseudoacacia*) (referred to as 'black locust'; Fig. 1E). The aboveground biomass of the trees was harvested using a forage harvester in February 2015, March 2018, and February 2021. Tree harvests were conducted when the soil was frozen to avoid soil compaction by the machinery.

The management of the crop rows was identical to that of the monoculture cropland. The crop rotation was maize (*Zea mays*) – summer barley (*Hordeum vulgare*) – summer oat (*Avena strigosa*) – winter wheat (*Triticum aestivum*) – winter barley (*H. vulgare*) (2018 – 2019 – 2020 – 2021 – 2022). In March 2022, the crop row of the agroforestry system and the monoculture cropland received 40 – 0 – 0 kg N – P – K ha^{-1} in the form of mineral fertilizer. Maize and small-grain cereal crops were harvested with a standard 24-m wide combine harvester. Crop rows and monoculture cropland were conventionally ploughed at a depth of 25 cm.

It is well established that trees in alley-cropping systems introduce spatial heterogeneity (e.g. Guillot et al. 2021; Beule et al. 2020). Thus, samples in the agroforestry system were collected along four transects (replicate plots) per tree species spanning from the tree row into the crop row. Samples were collected in the center of the tree row as well as at 1 m, 7 m, and 18 m distance from the trees within the crop row (3 tree species × 4 transects × 4 sampling locations within each transect = 48 samples; Fig. 1B). In the adjacent monoculture cropland, samples were collected in the center of each replicate plot (4 samples; Fig. 1B).

Soil and earthworm sampling

Soil samples for the determination of the microbial community and general soil properties (soil pH, soil organic carbon (SOC), total N, extractable nutrients) in the upper 5-cm topsoil were collected on 23 February 2022 (prior to fertilization). Three soil samples with a volume of 250 cm^3 were collected at each sampling location using stainless steel cylinders (5 cm height), transferred to a sterile polyethylene bag, and thoroughly homogenized. An aliquot of approx. 50 g fresh soil was transferred into a sterile 50-ml Falcon tube and frozen at -20 °C in the field. Upon arrival at the laboratory, samples were stored at -20 °C until freeze-drying for 48 h. The freeze-dried material was

finely ground using a vortexer (Beule et al. 2019a) and stored at -20 °C until DNA extraction. The remaining fresh soil in the polyethylene bag was used for determination of the general soil properties. Since earthworm activity at our site is generally low in February, earthworm communities were sampled on 11 and 12 April 2022 as described below (see Extraction of earthworms). Soil samples (250 cm^3) for the determination on soil bulk density and water-filled pore space (WFPS) were collected on all sampling dates and sampling locations (see above).

Extraction of earthworms

Earthworms were directly extracted from soil by applying 5 L of 0.01% (w/v) allyl isothiocyanate (AITC) solution on an area of a quarter square meter as described by Zaborski (2003). The extractant was prepared in the field by adding 500 mg AITC (pre-dissolved in 50 ml isopropanol (w/v)) to 4.95 L tap water immediately before extraction. To ensure that the extractant was applied only to a surface of 0.25 m^2, it was poured into a 50 × 50 cm open metal frame which was embedded approx. 5 cm deep into the soil (Figure S1 A). To allow better monitoring of extracted worms, plant material within the frame was carefully removed prior to the application of AITC. Following the AITC application, the soil surface within the frame was continuously monitored for at least 30 min and any surfacing earthworms were collected using tweezers, thoroughly washed, and stored in tap water (Figure S1 B). Upon arrival to the laboratory, earthworms were stored at 5 °C in the dark until morphological identification. Morphological identification and recording of the fresh weight (including gut content) of each individual earthworm were carried out within 48 h post sampling and earthworms were subsequently released. Furthermore, earthworms were classified into three ecological groups: anecic, endogeic, and epigeic species, which were introduced by Bouché in 1972. Earthworm data have been deposited at the BonaRes repository (https://doi.org/10.20387/bonares-y3je-zz30).

Soil DNA extraction

DNA from soil was extracted using a CTAB-based protocol with phenol and chloroform/isoamyl alcohol extraction (Beule et al. 2019a). Briefly, 50 mg

finely ground soil was suspended in a mixture containing 1 ml CTAB, 1 μL 2-mercaptoethanol, and 2 μL pronase E (30 mg/ml). The mixture was incubated at 42 °C and subsequently at 65 °C for 10 min each and 900 μL phenol were added. The mixture was shaken, centrifuged at $4,600 \times g$ at room temperature for 10 min, and 800 μL of the supernatant were transferred into a new 2 mL tube. 800 μL chloroform/isoamyl alcohol (24:1 (v/v)) were added, the mixture was shaken, incubated on ice for 10 min, and centrifuged at $4,600 \times g$ at room temperature for 10 min. Following centrifugation, 700 μL of the supernatant were transferred into a new 2 mL tube, 700 μL chloroform/isoamyl alcohol were added, the mixture was shaken, incubated on ice for 10 min, and centrifuged at $4,600 \times g$ for 10 min at room temperature. DNA was precipitated by transferring 600 μL of the supernatant into a new 1.5 mL tube containing 200 μL PEG 6,000 (30% (w/v)) and 100 μL 5 M NaCl. The mixture was incubated at room temperature for 20 min and centrifuged at $20,240 \times g$ at room temperature for 15 min to pellet the DNA. DNA pellets were washed with 70% (v/v) EtOH twice and dried using a vacuum centrifuge. Dried pellets were re-dissolved in 50 μL $1 \times$ TE buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA); adjusted to pH 8.0 with HCl) and incubated at 42 °C for 2 h to facilitate re-dissolving. Extracted DNA was inspected using gel electrophoresis on 1% (w/v) agarose gels stained with SYBR Green Solution I (Thermo Fisher Scientific, Waltham, MA, USA).

Real-time PCR

Real-time PCR assays for the quantification of total bacteria, total fungi, Basidiomycota, Ascomycota, Acidobacteria, Actinobacteria, Alpha-, Beta-, Gammaproteobacteria, Bacteriodetes, and Firmicutes were performed in a Peqstar 96Q thermocycler (PEQLAB, Erlangen, Germany). The composition of the mastermix and the thermocycling conditions correspond to those described by Beule et al. (2020). Total archaea were amplified using the primer pair ARC787F and ARC1059R (Yu et al. 2005). The reaction mixture comprised of 3 μL mastermix (double distilled H$_2$O, buffer (10 mM Tris–HCl, 50 mM KCl, 2.0 mM MgCl$_2$, pH 8.3 at 25 °C); 100 μM of each deoxynucleoside triphosphate (New England Biolabs, Beverly, Massachusetts, USA); 0.3 μM of each primer;

$0.1 \times$ SYBR Green I solution (Thermo Fisher Scientific, Waltham, MA, USA); 1 μg μL^{-1} bovine serum albumin; 0.03 u μL^{-1} Hot Start *Taq* DNA Polymerase (New England Biolabs, Beverly, Massachusetts, USA)) and 1 μL of template DNA or double distilled H$_2$O for a negative control. Thermocycling conditions were as follows. Initial denaturation at 95 °C for 2 min followed by 40 cycles of denaturation (95 °C for 20 s), annealing (62 °C for 30 s), and elongation (68 °C for 20 s). Final elongation was performed at 68 °C for 5 min. Following amplification, melting curves were generated by step-wise heating of the PCR product from 65 to 95 °C by 0.5 °C per step under continuous fluorescence measurement. DNA extracts were tested for PCR inhibitors according to Guerra et al. (2020) and diluted 1:50 (v/v) in double distilled H$_2$O prior to PCR to overcome PCR inhibition. Real-time PCR data have been deposited at the BonaRes repository (https://doi.org/10.20387/bonares-y3je-zz30).

Library preparation and amplicon sequencing of soil fungi

Sequencing libraries were prepared by amplifying the fungal ITS1 region using the primer pair ITS1-F_KYO2 (5'-TAGAGGAAGTAAAAGTCGTAA-3') (Toju et al. 2012) / ITS86R (5'-TTCAAAGAT TCGATGATTCA-3') (Vancov & Keen 2009). PCR reactions were carried out in 96-well plates in an Eppendorf Mastercycler EP Gradient S thermocycler (Eppendorf, Hamburg, Germany) in 25 μL reaction volume within one PCR run using the same mastermix for all libraries. The reaction volume comprised 18.75 μL mastermix and 6.25 μL template DNA diluted 1:50 (v/v) in double distilled H$_2$O or double distilled H$_2$O for negative a control. The mastermix contained double distilled H$_2$O, buffer (10 mM Tris–HCl, 50 mM KCl, 2.0 mM MgCl$_2$, pH 8.3 at 25 °C), 100 μM of each deoxynucleoside triphosphate (New England Biolabs, Beverly, Massachusetts, USA), 0.5 μM of each primer (ITS1-F_KYO2 / ITS86R), 1 mg mL^{-1} bovine serum albumin, and 0.03 u μL^{-1} Hot Start *Taq* DNA Polymerase (New England Biolabs, Beverly, Massachusetts, USA). Each primer was a mixture of primer with (50%) and without (50%) Illumina TruSeq adapters (5'-GACGTG TGCTCTTCCGATCT-3' for the forward primer and 5'-ACACGACGCTCTTCCGATCT-3' for the reverse

primer) at the 5`-end. The thermocycling conditions were as per (Beule and Karlovsky 2021a, b): initial denaturation (95 °C for 2 min), 3 touch-up cycles of denaturation (95 °C for 20 s), annealing (50 °C for 30 s), and elongation (68 °C for 60 s) followed by 25 cycles of denaturation (95 °C for 20 s), annealing (58 °C for 30 s), and elongation (68 °C for 60 s), and final elongation (68 °C for 5 min). Following thermocycling, successfulness of the amplification was confirmed by visualizing 2 μL of the PCR product on 1.7% (w/v) agarose gels stained with SYBR Green I solution (Thermo Fisher Scientific GmbH, Dreieich, Germany). Gel electrophoresis was performed at 4.6 V cm^{-2} for 60 min. Libraries were shipped to the facilities of LGC Genomics (Berlin, Germany) for a second amplification step using standard i7- and i5-sequencing adapters and Illumina sequencing. The second PCR was performed in 20 μL reaction volume containing 1 × MyTaq buffer (Bioline GmbH, Luckenwalde, Germany), 15 pmol of each forward and reverse i7- and i5- sequencing adapters, 2 μl of Bio-StabII PCR Enhancer (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), and 0.075 u μL^{-1} MyTaq DNA polymerase (Bioline GmbH, Luckenwalde, Germany).The thermocycling conditions were as follows: initial denaturation (96 °C for 1 min), 3 touch-up cycles of denaturation (96 °C for 15 s), annealing (50 °C for 30 s), and elongation (68 °C for 90 s) followed by 7 cycles of denaturation (96 °C for 15 s), annealing (58 °C for 30 s), and elongation (68 °C for 90 s), and final elongation (70 °C for 2 min). DNA quantity was assessed on agarose gels and indexed sequencing libraries were pooled. The multiplexed libraries were sequenced using on an Illumina MiSeq using V3 chemistry (2 × 300 bp) (Illumina, Inc., San Diego, CA, USA) at LGC Genomics, Berlin, Germany. Amplicon sequencing data have been deposited at NCBI's Short Read Archive (PRJNA885015).

Processing of amplicon sequencing data

Raw paired-end sequencing data were demultiplexed using Illumina's bcl2fast v. 2.20 (Illumina, San Diego, CA, USA) and sorted by their barcodes. Barcodes with more than two mismatches as well as one-sided and conflicting barcode pairs were excluded. Furthermore, Illumina sequencing adapters and primers (allowing three mismatches per primer) were clipped, whereas reads shorter than

100 bp were excluded. Afterwards, reads were processed in QIIME 2 version 2022.2 (Bolyen et al. 2019). Sequence quality was evaluated utilizing the 'q2-demux' plugin. Using DADA2 (Callahan et al. 2016), forward and reverse reads were truncated to 220 and 180 bp, respectively, quality filtered (allowing two expected errors per read), and merged. Moreover, chimeras and singletons were removed. Afterwards, reads were clustered into exact amplicon sequence variants (ASVs). ASVs were taxonomically classified against the UNITE database version 8.3 QIIME developer release (Abarenkov et al. 2021) using a scikit-learn Naive Bayes machine-learning classifier ('q2-fit-classifier-naive-bayes' and 'q2- classify-sklearn' plugin) in the 'balanced' configuration ([6,6]; 0.96) as suggested by Bokulich et al. (2018). After non-fungal ASVs were removed, 3,945,026 sequence counts remained. Sequence counts were normalized to 41,564 counts per sample using scaling with ranked subsampling (SRS) (Beule and Karlovsky 2020) in the R environment v. 4.2.1 (R Core Team 2013) utilizing the 'SRS' R package version 0.2.3 (Heidrich et al. 2021). SRS curves were generated using the same R package. The normalized dataset contained 2,949 fungal ASVs.

Soil properties

Soil pH was determined from sieved (< 2 mm) and air-dried soil in deionized H$_2$O at a ratio of 1:2.5 (soil/water (w/v)). Soil bulk density was determined from 250-cm^3 soil cores dried at 105 °C for 24 h. WFPS was determined from the same sample assuming a particle density of 2.65 g cm^{-3}. Double lactate-extractable phosphorus (P$_{DL}$) and potassium (K$_{DL}$) were determined from sieved (< 2 mm) and air-dried soil as per (VDLUFA 1991a). Calcium chloride-extractable magnesium (Mg$_{CaCl2}$) was determined as described previously (VDLUFA 1991b). Carbonates in sieved (< 2 mm) and air-dried soil were removed using acid fumigation as per Harris et al. (2001) and concentrations of SOC and total nitrogen were determined on a CNS elemental analyzer (Vario EL Cube, Elementar, Germany). Soil properties data have been deposited at the BonaRes repository (https://doi.org/10.20387/bonares-y3je-zz30).

Statistical analysis

For each tree species, differences in soil properties, microbial abundance, and earthworm parameters among the different sampling locations within the agroforestry system (i.e. tree row, 1 m, 7 m, and 18 m into the crop row) and the monoculture cropland were tested using one-way analysis of variance (ANOVA) followed by Tukey honestly significant difference (HSD). Relationships between parameters (i.e. soil properties, microbial abundance, and earthworm parameters) were explored using Spearman's rank correlation test. For the soil fungal community, alpha (Shannon diversity, Chao1 index, and Pielou's evenness) and beta diversity (Bray–Curtis dissimilarity index) were computed using 'vegan' R package v. 2.6.2 (Oksanen et al. 2013). Differences in fungal community composition among the different sampling locations within the agroforestry system and the monoculture cropland were identified using permutational multivariate analysis of variance (PERMANOVA) based on Bray–Curtis dissimilarities using the 'vegan' R package. Multivariate homogeneity of dispersions was tested using the same R package. Furthermore, differential abundance analysis using data collapsed at genus level was performed

using the 'metacoder' R package v. 0.3.5 (Foster et al. 2017). The same package was utilized to generate heat trees. For each tree species, differences in alpha diversity and taxonomic groups of fungi among the different sampling locations within the agroforestry system and the monoculture cropland were tested using one-way ANOVA followed by Tukey HSD test or Kruskal–Wallis test followed by Dunn's test. All data were tested for normality of residuals (Shapiro–Wilk test) and homogeneity of variance (Levene's test). Statistical significance was considered at $p < 0.05$. All tests were performed in R version 4.1.2.

Results

Mean earthworm density in the tree rows of the agroforestry system was 2.9 to 12.3 times greater than in the crop rows and the monoculture cropland (Fig. 2A) ($p \leq 0.0216$). Likewise, mean earthworm biomass was 5.7 to 34.8 times greater in the tree rows than in the crop rows and the monoculture cropland (Fig. 2B) ($p \leq 0.0014$). A gradual decline in earthworm density from the trees into the crop rows and monoculture cropland was evident for all three tree species (Fig. 2A). For example, earthworm density was
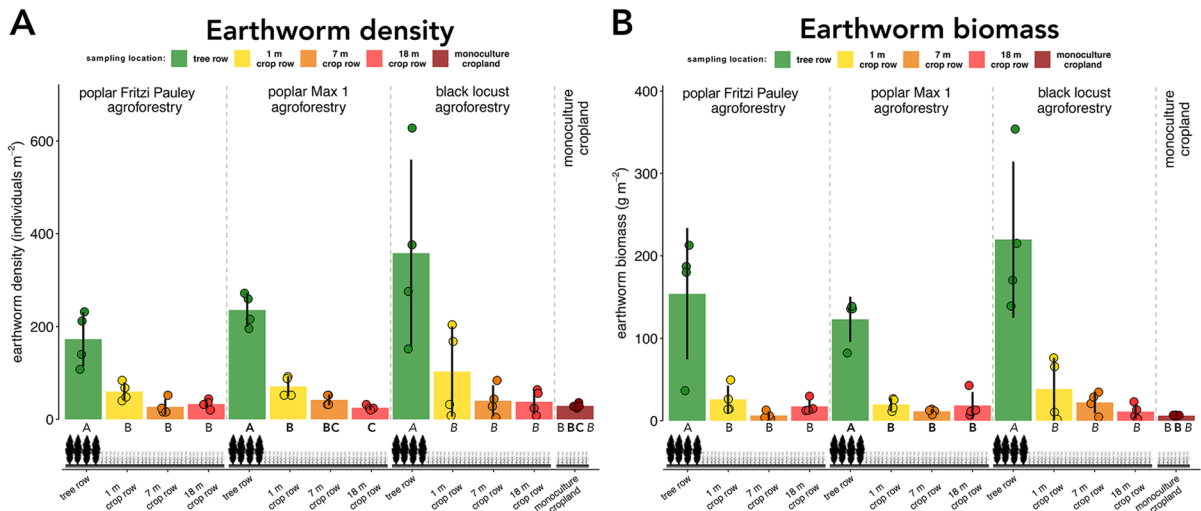

Fig. 2 Earthworm density (**A**) and biomass (**B**) in a paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agroforestry system. Within the agroforestry system, samples were collected in the tree row as well as at 1 m, 7 m, and 18 m distance from the tree row within the crop row. Bars with error bars represent the mean and its standard deviation ($n = 4$). Dots represent individual data points. Different uppercase letters of the same font indicate statistically significant differences ($p < 0.05$)

greater at 1 m than at 18 m distance from the trees and monoculture cropland under poplar Max 1 cultivation (Fig. 2A) ($p = 0.0344$). The gradual decline in population density was mainly driven by endogeic (Figure S2 A) rather than anecic species (Figure S2 C). Across sampling locations, population density and biomass of earthworms were strongly positively correlated ($r = 0.84$; $p < 0.0001$). In total, five different earthworm species of three different ecological groups were found at our study site: *Allolobophora chlorotica*, *Aporrectodea caliginosa*, *Aporrectodea rosea* (endogeic species), *Lumbricus rubellus* (epigeic species), and *Lumbricus terrestris* (anecic species). The monoculture cropland was dominated by endogeic species with no anecic and epigeic species present (Fig. 3). Anecic species were always present in the tree rows of all three tree species ranging from 13.8 to 44.9% of the total community and were
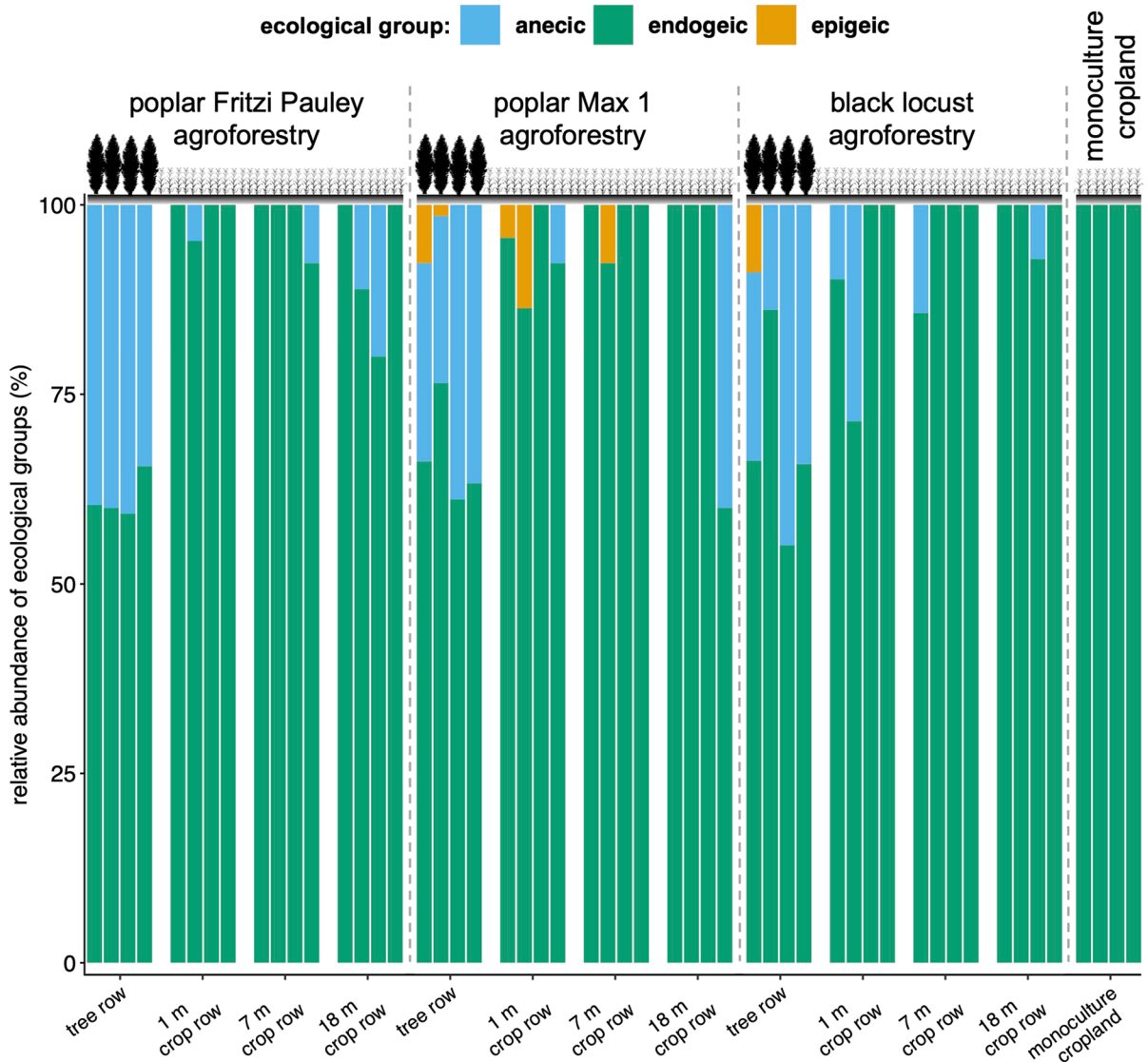

Fig. 3 Relative abundance of ecological groups of earthworms in a paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agroforestry system. Within the agroforestry system, samples were collected in the tree row as well as at 1 m, 7 m, and 18 m distance from the tree row within the crop row. Bars represent individual samples ($n = 4$)

occasionally recovered in the crop rows (Fig. 3). In the poplar Max 1 system, the epigeic species (i.e. *L. rubellus*) was found in half of all replicate plots in the tree row and at 1 m distance from the trees as well as in a quarter of all the replicate plots at 7 m distance (Fig. 3). Epigeic earthworms were only detected in one replicate plot of the tree rows in the black locust system (Fig. 3).

We quantified 12 groups of soil microorganisms (total bacteria, total fungi, total archaea, Basidiomycota, Ascomycota, Acidobacteria, Actinobacteria, Alpha-, Beta-, Gammaproteobacteria, Bacteriodetes and Firmicutes) and found only positive or no effects of agroforestry on microbial abundance. Total soil bacterial abundance was greater in the tree rows than at 18 m distance from the trees within the crop rows (Fig. 4A) ($p \leq 0.0378$). In the poplar Max 1 system, gene abundance of bacteria as well as Actinobacteria was greater under the trees as compared to 7 and 18 m into the crop row and the monoculture cropland (Fig. 4A, C) ($p \leq 0.0288$). In the same system, greater abundance of Acidobacteria and Bacteroidetes was detected in the tree row as compared to the crop row and the monoculture cropland (Fig. 4B, S3 A) ($p \leq 0.0290$) and Firmicutes were more abundant under the trees than at 18 m distance into the crop row and the monoculture cropland (Figure S3 B) ($p \leq 0.0092$). The abundance of Basidiomycota in the poplar Max 1 system was greater in the tree row than in the crop row at 7 and 18 m distance and in the monoculture cropland (Figure S4 C) ($p \leq 0.0313$).

Gene abundances of total soil fungi, Ascomycota, total archaea, Alpha-, Beta-, and Gammaproteobacteria did not differ among sampling locations (Figure S4 A-B, S5, S6).

The soil fungal community was dominated by Ascomycota ($51.4 \pm 16.7\%$), Basidiomycota ($32.4 \pm 22.4\%$), and Mortierellomycota ($5.8 \pm 4.0\%$). The dominant fungal classes were Sordariomycetes ($28.7 \pm 14.4\%$), Agaricomycetes ($27.4 \pm 22.8\%$), Dothideomycetes ($6.9 \pm 5.3\%$), and Mortierellomycetes ($5.8 \pm 4.0\%$) (Fig. 5A). Acremonium ($8.6 \pm 6.2\%$), Mortierella ($4.9 \pm 3.4\%$), Coprinellus ($4.5 \pm 8.9\%$), Laccaria ($3.2 \pm 9.8\%$), and Agrocybe ($2.9 \pm 10.7\%$) were the most abundant genera in the dataset. Alpha diversity (Shannon diversity, Chao1 index, and Pielou's evenness) did not differ among sampling locations (i.e. tree row, 1, 7, 18 m crop rows, and monoculture cropland) within each agroforestry system (i.e. poplar Fritzi Pauley, poplar Max 1, and black locust agroforestry system) (Table S1). Sampling location significantly affected fungal community composition (Table S2) ($p = 0.0001$) and was driven by the differences between the tree row and the arable land (crop row and monoculture cropland) (Fig. 5B). Multivariate homogeneity of dispersions was given under all PERMANOVA test conditions performed ($p \geq 0.32$). Differential abundance analysis was visualized by heat trees (Figure S 7) and a total of 15 genera and species were identified whose relative abundance was either positively or negatively affected by agroforestry (Fig. 6). Relative abundance
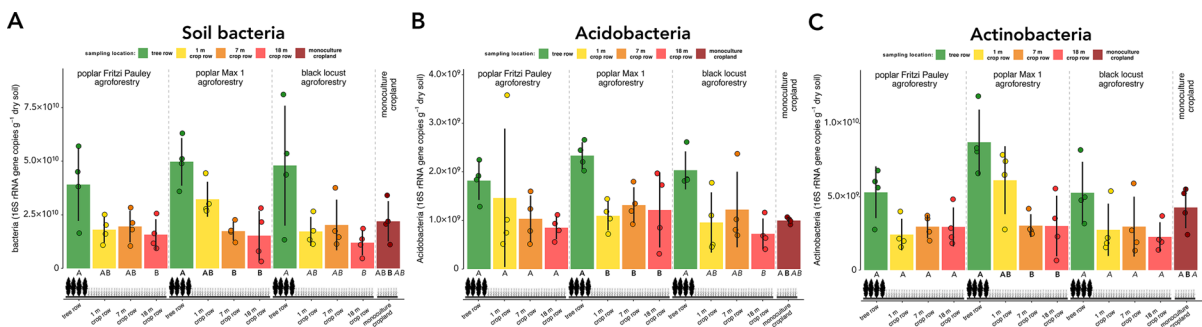

Fig. 4. 16S rRNA gene abundance of total bacteria (**A**), Acidobacteria (**B**), and Actinobacteria (**C**) in soil of a paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agroforestry system. Within the agroforestry system, topsoil samples were collected in the tree row as well as at 1 m, 7 m, and 18 m distance from the tree row within the crop row. Bars with error bars represent the mean and its standard deviation ($n = 4$). Dots represent individual data points. Different uppercase letters of the same font indicate statistically significant differences ($p < 0.05$)
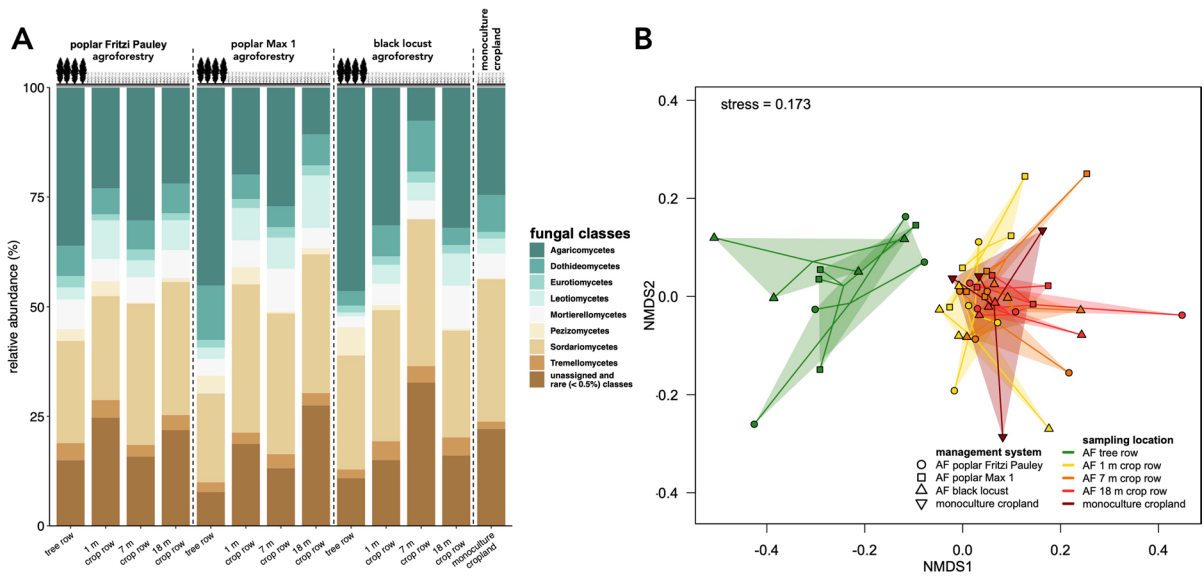
Fig. 5 Soil fungal community composition in a paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agroforestry system. Within the agroforestry system, soil samples were collected in the tree row as well as at 1 m, 7 m, and 18 m distance from the tree row within the crop row ($n = 4$). Relative abundances of dominant ($\geq 0.5\%$ mean relative abundance) fungal classes (**A**) are shown. Beta diversity is shown by non-metric multidimensional scaling (NMDS) of Bray–Curtis dissimilarities (**B**). Circles, squares, and triangles represent individual data points. AF = agroforestry

of *Gamsia, Ilyonectria, Laccaria,* and *Preussia* spp. were generally enhanced by the tree rows, whereas the increased relative abundance of *Inocybe* spp. was specific for poplar Max 1 tree rows (Fig. 6). Relative abundance of *Protomyces* spp., *Sporobolomyces* spp., and *Blumeria graminis* showed a positive trend with increasing distance from the trees. Among these, *Blumeria graminis* was absent in the tree rows. Relative abundance of *Mycosphaerella tassiana* as well as *Dioszegia, Itersonilia, Lectera, Leucosporidium, Microdochium,* and *Neosetophoma* spp. was overall higher in the crop row or monoculture cropland compared to the tree rows; however, no trends regarding sampling distance were observed (Fig. 6).

In the black locust system, SOC and total N concentrations in topsoil (upper 0–5 cm soil) were 54 to 97% greater in the tree rows than in the crop row and monoculture cropland (Fig. 7) ($p \leq 0,0004$). Likewise, in the poplar Max 1 system, SOC concentrations in topsoil were greater in the tree rows than in the crop row and monoculture cropland (Fig. 7A) ($p \leq 0.0376$). Tree rows of poplar Fritzi Pauley showed greater SOC concentrations than the crop row at 18 m distance (Fig. 7A) ($p = 0.0093$) but

similar total N concentrations among all sampling locations (Fig. 7B). Soil bulk density showed slight differences across sampling locations, however, no distinct spatial pattern among sampling locations was detected (Table S3). In the black locust system, Mg_{CaCl2} was greater under the trees than in the crop row ($p = 0.0001 - 0.0004$) (Table S3). Similarly, Mg_{CaCl2} in the system with poplar Fritzi Pauley was greater in the tree row than in the crop row and monoculture cropland ($p = 0.0006 - 0.0086$) (Table S3). In both, the poplar Max 1 and the black locust system, K_{DL} increased in the tree row as compared to the crop row and monoculture cropland ($p < 0.0001 - 0.0073$) (Table S3). Tree rows of poplar Max 1 showed greater P_{DL} as compared to monoculture cropland ($p = 0.0106$). Earthworm density and biomass were positively correlated to SOC and total N concentrations, K_{DL}, Mg_{CaCl2}, and WFPS ($r = 0.35 - 0.56$; $p < 0.0001 - 0.011$) but not P_{DL} ($r = 0.04 - 0.14$; $p = 0.32 - 0.77$) (Figure S8). Positive relationships were found between soil bacteria, Bacteroidetes, Acidobacteria, and Actinobacteria and SOC and total N concentrations, K_{DL}, Mg_{CaCl2}, and P_{DL} ($r = 0.28 - 0.54$; $p < 0.0001 - 0.041$) (Figure S9).
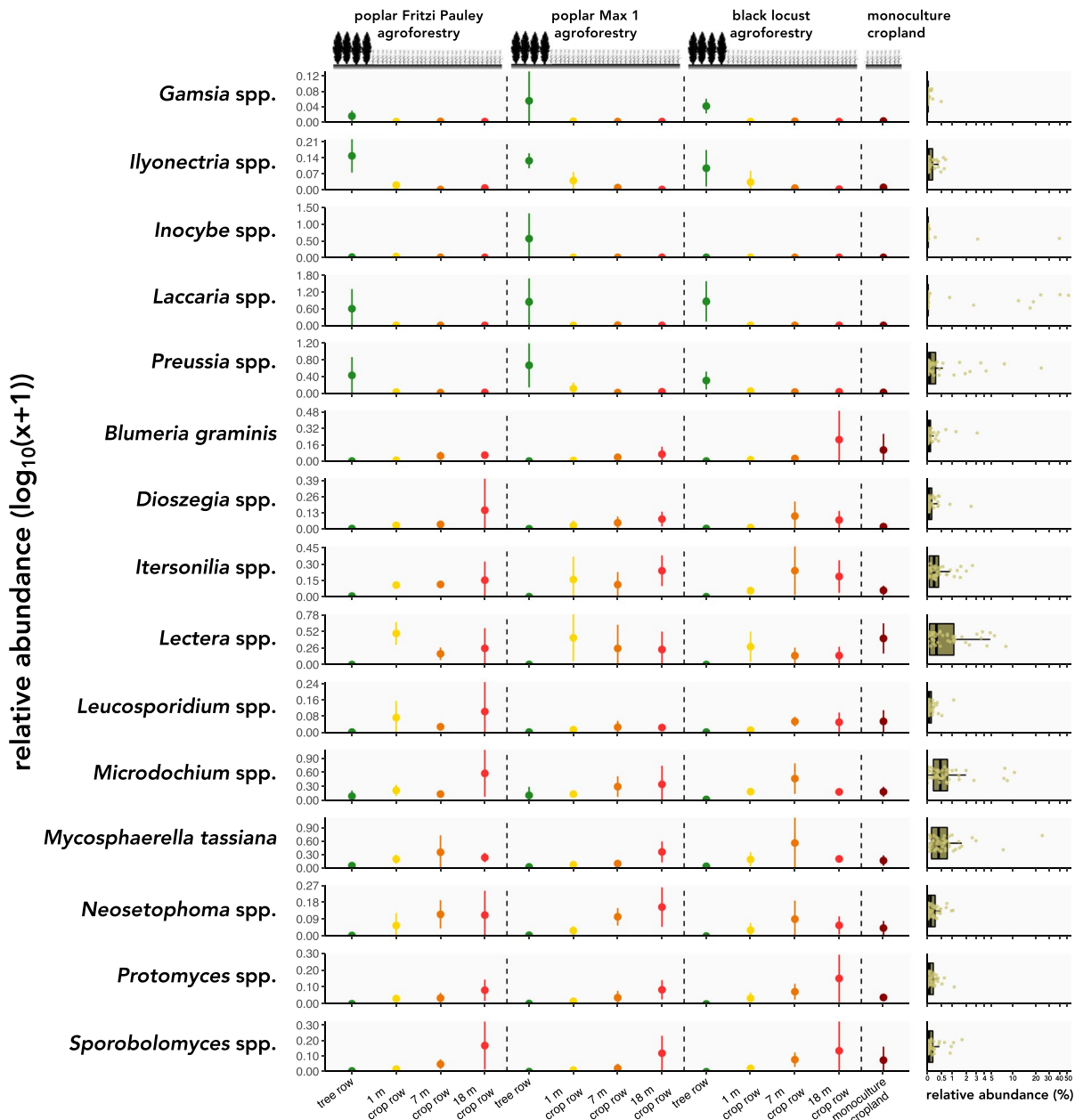
Fig. 6 Differentially abundant genera and species in a paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agrofor-estry system. Within the agroforestry system, soil samples were collected in the tree row as well as at 1 m, 7 m, and 18 m distance from the tree row within the crop row ($n=4$)

Discussion

Improved soil fertility through agroforestry

In agreement with previous findings (e.g. Pardon et al.

2017), soil fertility (i.e. SOC, total N, and extractable nutrients (K_{DL} and Mg_{CaCl2})) increased under the trees (Fig. 7, Table S3). This increase was likely due to the input of above- and belowground tree litter-derived nutrients, which declines with increasing distance
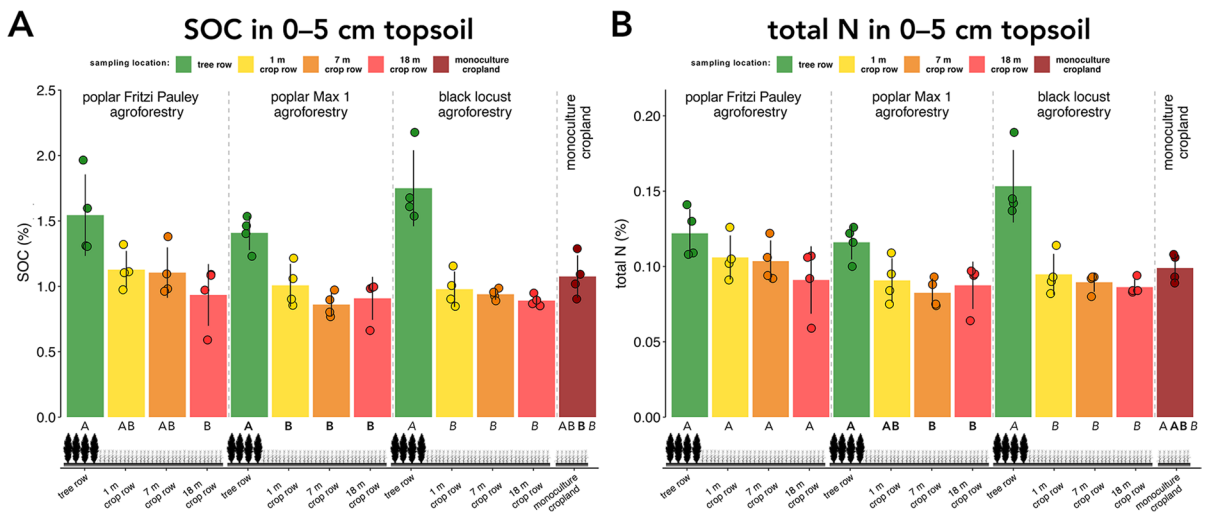
Fig. 7 Soil organic carbon (SOC) (A) and total N concentrations (B) in 0–5 cm topsoil in a paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agroforestry system. Within the agroforestry system, topsoil samples were collected in the tree row as well as at 1 m, 7 m, and 18 m distance from the tree row within the crop row. Bars with error bars represent the mean and its standard deviation ($n=4$). Dots represent individual data points. Different uppercase letters of the same font indicate statistically significant differences ($p<0.05$)

from the trees (Schmidt et al. 2021). Although tree-derived nutrient inputs can reach several meters into the crop row (Schmidt et al. 2021), increased soil fertility did not gradually extend into the crop rows which we attribute to the relatively young age (12 years) of our system (*cf.* Pardon et al. 2017). We expect that as our system ages and more tree litter is deposited, the increase in soil fertility under the trees will increasingly extend into the crop rows.

Agroforestry promotes earthworm communities

In the present study, earthworms were sampled using AITC extraction without hand sorting. Compared to hand sorting combined with AITC extraction (e.g. as per ISO (2018) 23611–1), using exclusively AITC extraction for earthworm sampling may result in reduced efficacy for juveniles and endogeic species whereas adult anecic earthworms are well recovered (e.g.Pelosi et al. 2009; Čoja et al. 2008). Hand sorting, however, is rarely feasible for large-scale studies as it requires a substantial amount of labour and may prolong the sampling campaign (Iannone et al. 2012). Furthermore, if additional on-site data needs to be collected, chemical extraction is preferred over hand sorting since it does not physically disturb the soil (Iannone et al. 2012; Lees et al. 2016; Tóth et al. 2020).

Unlike Price and Gordon (1998), we did not detect tree-species effects on earthworm abundance, diversity or community composition at our study site. In line with previous studies (Cardinael et al. 2019; D'Hervilly et al. 2022), population size of earthworms was greater in the tree rows as compared to the crop rows (Fig. 2A). In agreement with the results of Cardinael et al. (2019), the increase in density was evident for all three ecological groups (Figure S2). Furthermore, we were able to demonstrate that earthworm abundance decreases with increasing distance from the trees (Fig. 2A). This gradual decline was driven mainly by endogeic species (*cf.* Figure S2 A, C, E), which almost exclusively colonize the topsoil. A recent study conducted in two similarly managed alley-cropping agroforestry systems of similar age in Germany found that tree roots in the topsoil of the tilled crop rows are scarce (Schmidt et al. 2021). Consequently, endogeic species in the crop row likely benefited from tree litter input in vicinity of the trees rather than tree root litter. To our best knowledge, the present study is the first to investigate earthworm communities at more than two distances from the tree rows into the crop rows. Although a greater number

of sampling locations is demanding, we believe that such study designs are essential to improve the characterization of spatial gradients within agroforestry systems, and thus, the extent by which the tree rows influence the crop rows and vice versa.

Earthworm communities in the tree rows were characterized by a shift towards anecic earthworms (Fig. 3), which is in agreement with previous findings (D'Hervilly et al. 2020, 2021). In contrast to the gradual decrease of endogeic earthworms with increasing distance from the trees, density of anecic earthworms did not decline gradually but rapidly dropped which is most likely due to the tillage in the crop rows. It is well established that anecic earthworms benefit from reduced tillage (Chan 2001; Ernst & Emmerling 2009). Unsurprisingly, the absence of tillage under the trees has recently been identified as a main driver for the abundance increase of anecic earthworms in the tree rows of agroforestry systems (Cardinael et al. 2019). Since anecic earthworms feed on the soil surface, litter fall and understory vegetation should also be considered as promoting factors for deep burrowing species. Additionally, Gilbert et al. (2014) were able to show that saprophagous earthworms, including *Lumbricus terrestris*, benefit from tree fine roots and fine root associated-mycorrhizae as a feeding source. In order to disentangle the impact of soil management (i.e. tillage) and food resource availability (i.e. litter, fine roots, mycorrhizae, and understory vegetation) on the abundance of anecic earthworms, studies in no-till agroforestry systems are required.

The sporadic detection of epigeic individuals identified as *L. rubellus* under as well as in close proximity to the trees (Figure S2 E, F) is in accordance with the results of D'Hervilly et al. (2020). We argue that this is likely due to tree litter input, as those species require a permanent surface cover with organic material and are therefore mostly absent in arable croplands. We speculate that depending on the size of the agroforestry system, the colonization of tree rows by epigeic earthworms requires several years to reach a spatially homogeneous equilibrium state.

Effects of agroforestry on soil microbial population size

The population size of total bacteria, Acidobacteria, and Actinobacteria was greater in the vicinity of trees in all agroforestry systems (Fig. 4), which is in

line with results of recent studies (Beule et al. 2020; Guillot et al. 2021). The positive effect of trees in agroforestry systems on the abundance of soil microorganisms is well-described (Banerjee et al. 2016; Beuschel et al. 2019; Beule et al. 2020; Guillot et al. 2021; Luo et al. 2022) and is likely due to differences in soil management (i.e. absence of tillage in the tree rows versus tilled crop rows) and vegetation cover (i.e. woody perennials versus annual crops) (Beule et al. 2022a). Furthermore, some studies were able to demonstrate that microbial abundance increases with decreasing distance from the trees (e.g. Guillot et al. 2019, Beule et al. 2020; D'Hervilly et al. 2021; Luo et al. 2022). At our study site, no such trends were observed except for total bacteria (Fig. 4A), Actinobacteria (Fig. 4C), Firmicutes (Figure SI2 B), and Bacteriodetes (Figure SI 3C) in the poplar Max 1 system. In contrast to previous studies where trees were either not harvested at all (Guillot et al. 2019, 2021; D'Hervilly et al. 2021) or harvested four to five years (Beule et al. 2020; Luo et al. 2022) prior to soil sampling, the aboveground biomass of the trees at our study site was harvested one year prior to soil sampling. Therefore, we suggest that tree harvesting temporally alters soil microbial communities throughout agroforestry systems. We relate these alterations to changes in substrate input due to reduced tree litter input and/or altered tree root functioning (e.g. dying off of roots, changes in quantity and quality of root exudation). Furthermore, this hypothesis may explain why in contrast to previous studies (Beuschel et al. 2019; Beule et al. 2020; Guillot et al. 2021), soil fungal abundance at our study site was not promoted by agroforestry (Figure SI 3). Further studies exploring temporal dynamics along tree rotation cycles are required to investigate this hypothesis.

Agroforestry alters the soil mycobiome and putative phytopathogen abundance

Our finding that agroforestry does not affect alpha (Table S1) but beta diversity of soil fungi (Table S2, Fig. 5B) agrees with previous studies on the soil mycobiome of agroforestry systems (Beule et al. 2021, Beule & Karlovsky 2021b). Differences in fungal community composition were mainly driven by the tree rows (Fig. 5B) which are known to exert strong influence on the soil fungal community even few months after tree planting (Beule & Karlovsky

2021b). Across all three tree species, the tree-row associated mycobiome was characterized by the promotion of genera harbouring ectomycorrhizal fungi (EMF) such as *Laccaria* and *Preussia* spp. (Fig. 6). Notably, affiliates of the genus *Inocybe*, harbouring EMF that can associate with poplar (e.g. Long et al. 2016), were strongly promoted under poplar Max 1 (Fig. 6) which indicates a tree species-specific tree-EMF interaction. Colonization of roots by mycorrhiza is often advantageous for plant growth as it improves nutrient and water acquisition. Under field conditions, poplar and black locust trees can associate with both arbuscular (AMF) as well as ectomycorrhizal fungi (e.g. Khasa et al. 2002; Bratek et al. 1996). For poplar trees, colonization rates of AMF and EMF have been shown to be poplar genotype-specific (Khasa et al. 2002), whereas black locust genotype specificity of mycorrhizal associations has not been investigated yet. Our findings on EMF highlight the importance of investigating several tree species (e.g. hardwood and fast-growing tree species) at the same study site in order to disentangle tree-species from study-site effects.

Concerns regarding an increased risk of crop diseases is among the main impediments of farmers to implement temperate agroforestry. In our study, a gradual decrease in relative abundance of *Blumeria graminis* (formerly known as *Erysiphe graminis*) with decreasing distance from tree rows was observed (Fig. 6). *B. graminis* is the causal agent of powdery mildew, one of the most common cereal diseases worldwide that can reduce grain quality and cause significant yield losses in temperate zones (e.g. Bélanger et al. 2002; Dreiseitl 2011; Dean et al. 2012). The ability of *B. graminis* to cause disease is host specific and thus *forma specialis* dependent (Wyand and Brown 2003). Sequencing of short-read amplicons (2×300 bp) did not enable identification of *B. graminis* on *forma specialis* level, which is not surprising considering the limited taxonomic resolution of short-read amplicon sequencing (Heidrich & Beule 2022). Furthermore, *B. graminis* can overwinter in form of cleistothecia on plant residues (Zhang et al. 2005). Thus, it was not possible to relate *B. graminis* to any of the host crops that were grown in the crop rotation at our study site. Hence, we refer to *B. graminis* as a putative pathogen. Still, our results provide the first evidence of a positive effect of tree rows in temperate agroforestry systems on the reduction of the putative pathogen *B. graminis*. As microbial

antagonists of *B. graminis* have been isolated in Germany (Köhl et al. 2019), antagonistic interactions in soil and on aboveground crop parts may have contributed to the suppression of *B. graminis* at our study site. In addition to microbial antagonism, the ability of earthworms to suppress soil-born fungal phytopathogens is well recognized (e.g. Plaas et al. 2019; Stephens et al. 1994). Thus, the gradual increase in endogeic earthworm abundance with decreasing distance from the trees may have enhanced biological control within the crop rows and thereby contributed to the suppression of *B. graminis*.

In addition to potential biological control, tree rows in agroforestry systems increase structural diversity and act as physical barriers which is expected to lower the spread of crop diseases through the dilution of the host crop (host dilution effect) (Beule et al. 2019b). In 2019, Kanzler et al. reported reduced wind speed and evaporation rates at our study site under the agroforestry as compared to the monoculture cropland. Furthermore, reductions of wind speed and evaporation were dependent on the distance from the trees (Kanzler et al. 2019). Such microclimatic alterations are known to affect the epidemiology of plant diseases (e.g. Aust & von Hoyningen-Huene 1986, Waggoner 1965). Overall, the enhanced control of *B. graminis* in the agroforestry as compared to the monoculture cropland system cannot be attributed directly to one of the factors listed above. We rather expect that the control of *B. graminis* was due to a combination of factors that cannot be disentangled in our experimental setting.

As of writing, the study conducted by Beule et al. (2019b) is the only study that investigated the effect of temperate agroforestry on crop health. Their results revealed that colonization of oilseed rape plants with *Verticillium longisporum* and wheat grain with *Fusarium tricinctum* was lower in temperate agroforestry systems compared to monoculture cropland systems. Colonization of wheat and barley grain and oilseed rape plants with other major fungal pathogens did not differ between agroforestry systems and monoculture systems (Beule et al. 2019b). Furthermore, the authors observed a relationship between abundance of a phytopathogen and distance to the trees only for the phytopathogen *Leptosphaeria biglobosa* in oilseed rape plants. Considering our findings on *B. graminis,* we hypothesize that suppression of fungal phtyopathogens within agroforestry

systems is limited to certain pathogens and is a function of the distance to the trees (i.e. suppressiveness increases as the distance to the trees decreases). Studies on diseases and disease related factors in temperate agroforestry systems are scarce. We suggest that future research should investigate crop diseases in agroforestry systems of different spatial designs (e.g. wide versus narrow crop allays) as well as management practices. New findings could help farmers to optimize the design of future agroforestry systems in order to maximize the beneficial effects of these systems on disease control.

Conclusion

The integration of tree rows into arable land (agroforestry) increased the abundance of soil bacteria and earthworms (anecic, endogeic, and epigeic species) as compared to monoculture cropland. We attribute this mainly to the absence of tillage and the input of large amounts of tree litter under the trees. Community composition of soil fungi was altered by the tree rows, resulting in a tree-row associated mycobiome, which was particularly characterized by an increased proportion of EMF. The tree-row associated mycobiome not just enhances overall fungal diversity of agroforestry systems but is also expected to alter soil functions such as nutrient cycling. As the distance from the trees decreased, the proportion of *Blumeria graminis,* the causal agent of powdery mildew, decreased. We suggest that enhanced microbial antagonism, increased earthworm densities and/or altered microclimatic conditions contributed to the suppression of *B. graminis* within the agroforestry system. Whereas distinct tree-distance effects were observed, tree-species effects were identified as a minor driver of the abundance and composition of soil communities at our study site. Overall, agroforestry benefits the abundance, diversity, and function of soil biota and may enhance soil suppressiveness. Future research should investigate crop diseases in agroforestry systems of different spatial designs and management practices in order to maximize the beneficial effects of these systems on disease control.

Declarations

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