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ORIGINAL PAPER

Impact of nitrogen and phosphorus addition on resident soil and root mycobiomes in beech forests

S. Clausing[1] · L. E. Likulunga[1,2] · D. Janz[1] · H. Y. Feng[1,3] · D. Schneider[4] · R. Daniel[4] · J. Krüger[5] · F. Lang[5] · A. Polle[1]

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Abstract

N and P are essential macronutrients for all organisms. How shifts in the availability of N or P affect fungal communities in temperate forests is not well understood. Here, we conducted a factorial P × N fertilization experiment to disentangle the effects of nutrient availability on soil-residing, root-associated, and ectomycorrhizal fungi in beech (*Fagus sylvatica*) forests differing in P availability. We tested the hypotheses that in P-poor forests, P fertilization leads to enhanced fungal diversity in soil and roots, resulting in enhanced P nutrition of beech, and that N fertilization aggravates P shortages, shifting the fungal communities toward nitrophilic species. In response to fertilizer treatments (1×50 kg ha^{-1} P and 5×30 kg ha^{-1} N within 2 years), the labile P fractions increased in soil and roots, regardless of plant-available P in soil. Root total P decreased in response to N fertilization and root total P increased in response to P addition at the low P site. Ectomycorrhizal species richness was unaffected by fertilizer treatments, but the relative abundances of ectomycorrhizal fungi increased in response to P or N addition. At the taxon level, fungal assemblages were unaffected by fertilizer treatments, but at the order level, different response patterns for saprotrophic fungi among soil and ectomycorrhizal fungi on roots were found. Boletales increased in response to P, and Russulales decreased under N + P addition. Our results suggest that trait conservatism in related species afforded resistance of the resident mycobiome composition to nutritional imbalances.

Keywords Soil fertility · Mycorrhiza · Saprotrophic fungi · Stratification · Seasonality · Ecosystem nutrition

Introduction

Nitrogen (N) and phosphorus (P) are essential nutrients that determine plant growth and productivity in many terrestrial ecosystems (Elser et al. 2007; Vitousek et al. 2010).

✉ A. Polle
apolle@gwdg.de

1 Forest Botany and Tree Physiology, Georg-August Universität Göttingen, Büsgenweg 2, 37077 Göttingen, Germany

2 Biological Sciences Department, University of Zambia, Great East Road Campus, 32379 Lusaka, Zambia

3 Experimental Center of Forestry in Northern China, Chinese Academy of Forestry, Men Tougou District, Shuizha West Road 1, Beijing 102300, People's Republic of China

4 Genomic and Applied Microbiology & Göttingen Genomics Laboratory, Georg-August University Göttingen, Grisebachstr. 8, 37077 Göttingen, Germany

5 Soil Ecology, University of Freiburg, Bertoldstraße 17, 79085 Freiburg (i.Br.), Germany

The main natural sources of N and P are biological N fixation from atmospheric N_2 for N and rock weathering for P (Augusto et al. 2017; Wardle, 2004). Therefore, P availability is mainly dependent on the parent soil material and soil age (Augusto et al. 2017; Wardle, 2004), while N can also be influenced by anthropogenic inputs, for example, N emissions from agriculture and the burning of fossil fuels (Galloway et al. 2008). Forests are often naturally N-limited (Vitousek et al. 2010). Consequently, anthropogenic N deposition can change the nutrient regime and affect the P demand of forest ecosystems (Vitousek et al. 2010). N deposition into forest ecosystems has increased primary production in N-limited forest ecosystems (Du and De Vries, 2018; Schulte-Uebbing and De Vries, 2018). Increasing N:P ratios in plant tissues have therefore been suggested to indicate that many European forest ecosystems are transitioning from N to P limitation (Jonard et al. 2015; Peñuelas et al. 2013).

In temperate and boreal forest soils, large fractions of P and N are bound by organic matter and, thus, not directly available for uptake by trees (Lambers et al. 2008; van der Heijden et al. 2008). Trees benefit from P and

N mineralization by microbial decomposers (Baldrian, 2017; Schimel and Schaeffer, 2012). Soil fungi are generally more efficient degraders of complex plant compounds than other soil microbiota (López-Mondéjar et al. 2018; Štursová et al. 2012). Thus, the taxonomic diversity and functional composition of soil fungal microbiomes is of high relevance for forest P and N nutrition. Both saprotrophic and ectomycorrhizal fungi (EMF) contribute to N and P mobilization by secreting organic acids and producing hydrolytic and oxidative exoenzymes (Bödeker et al. 2014; Courty et al. 2010; Op De Beeck et al. 2018; Pritsch and Garbaye, 2011). In deciduous temperate forest soils, the fraction of EMF hyphal biomass is similar to or even higher than that of saprotrophs, suggesting that EMF have key functions in nutrient recycling in these ecosystems (Awad et al. 2019), though this is dependent strongly on the availability of nutrients (Högberg et al. 2017). Trees invest a higher proportion of carbon (C) into the symbiont when N is limiting, which stimulates fungal growth (Högberg et al. 2017; Näsholm et al. 2013). Therefore, the fungal N requirement increases, further decreasing N return to the plant. By this mechanism, the relative abundances of mycorrhizal and saprotrophic fungi may be shifted in favor of the former (Högberg et al. 2017). However, the impact of changes in N and P availability on these functional groups and the P nutrition of trees is still unknown.

Belowground fungal communities are affected by multiple abiotic and biotic habitat filters such as climate, geographic location, soil type, and vegetation that drive their composition (Bahnmann et al. 2018; Bahr et al. 2015; Goldmann et al. 2016; Kolaříková et al. 2017; Suz et al. 2014; Tedersoo et al. 2014; Wubet et al. 2012). Among these drivers, N is an important factor that affects fungi in soil (Almeida et al. 2019), fungi thriving on roots (Nguyen et al. 2020; Schröter et al. 2019), and fungal symbionts, i.e., EMF (Cox et al. 2010; de Witte et al. 2017; van der Linde et al. 2018). For example, Lilleskov et al. (2002) reported a shift in the EMF community in Alaska toward nitrophilic species and, thus, a loss in diversity along a gradient of increasing N deposition. In boreal spruce forests, N fertilization was shown to cause a significant turnover of soil fungal communities, decrease fungal biomass, and increase the N:P ratio of the needles (Allison et al. 2007; Almeida et al. 2019). Other studies reported only weak or no effect of N treatments on the fungal community composition (Maaroufi et al. 2019; Nicolás et al. 2017; Purahong et al. 2018), and relationships with P mobilization were not detected (Forsmark et al., 2021). Empirical studies and theoretical models suggest that EMF in temperate beech forests are less sensitive to N deposition than conifers (Lilleskov et al. 2019; Rotter et al. 2020; Taylor et al. 2000), but it is unknown how N fertilization affects EMF and other soil fungi when N availability is increased under P shortage.

Only a few studies have investigated fungal communities after P fertilization in forests. Almeida et al. (2019) reported significant community turnover and loss of fungal biomass after P fertilization in a spruce forest. However, along a natural P gradient in temperate beech forests, EMF diversity increased with increasing P availability (Zavišić et al. 2016). After the addition of superphosphate to P-limited young beech trees, the EMF community composition was altered, but microbial biomass was unaffected (Zavišić et al. 2018). These disparate observations indicate that the responses of EMF and other fungi to P inputs depend on the P supplied by the soil and likely the interaction of P and N supply (de Witte et al. 2017). Furthermore, different availabilities of carbon (C) for fungal communities colonizing the root surface and those living in soil (Clausing et al. 2021; Clemmensen et al. 2013) may lead to divergent responses of fungi to N and P availabilities in each of these habitats. A further important aspect underlying fungal responses to changes in nutrient resources is their phylogenetic relationship because fungal traits for nutrient acquisition are relatively conserved within a phylum or subphylum (Treseder and Lennon, 2015) and therefore shifts in phylogenetically related fungi in response to enhanced N or P availabilities might be expected. However, experiments addressing these ecological processes are scarce (Amend et al. 2016; Zanne et al. 2020).

The aim of our study was to investigate how shifts in N and P availability affect fungal assemblages and functional composition with different degrees of reliance on root and soil nutrient resources. To this end, we studied the richness, diversity, and taxonomic composition of EMF living in symbioses with root tips and conducted Illumina sequencing of whole, root-associated fungal (RAF), and soil-associated fungal (SAF) assemblages in beech forest plots fertilized with either P, N, or P + N and untreated plots (*Fagus sylvatica* L.). Since soil fertility affects fungal assemblages, we selected three forests differing strongly in P availability (Lang et al. 2017) and analyzed the responses of the fungal communities and root N and P contents to fertilization in the organic layer and mineral soil. Since the EMF community composition and richness in P-rich soils of our selected forest sites differed from that in P-poor soil (Zavišić et al., 2016), we hypothesized that P fertilization on P-poor soil results in increased P availability and shifts the composition of EMF and RAF toward those of fungal assemblages in P-rich soil. Thereby, the abundance of mycorrhizal fungi increases relative to that of saprotrophic fungi, leading to higher P nutrition of roots. Second, we hypothesized that P fertilization has no effects on EMF, RAF, or SAF richness, diversity, community composition, or root P contents in P-rich soil. Third, we proposed that N fertilization results in higher inorganic N availability, which shifts the mycorrhizal fungal community composition toward nitrophilic

fungal phyla in P-rich soil. Since these fungi are usually less species-rich and produce less mycelium (Lilleskov et al. 2019; Taylor et al. 2000), we expected a higher abundance of saprotrophic fungi relative to mycorrhizal fungi in the soil. Fourth, we hypothesized that in poor soil, N fertilization aggravates P shortages in roots and, therefore, EMF richness, diversity, and composition are unaffected or increase as trees maintain investment in mycorrhizas to counteract P deficiency.

Material and methods

Site characteristics and study plots

The N and P fertilization experiments were carried out in three beech (*F. sylvatica* L.) forests, differing in parent material and thus, soil P stocks. The high-P site (HP) Bad Brückenau is located in the biosphere reservation "Bayerische Rhön" on basalt, the medium-P site (MP) Mitterfels is situated in the Bavarian Forest on paragneiss and the low-P site (LP) Luess is located in the North German Plain on sandy till. P stocks in the A horizon (1 m soil depth) at the HP, MP, and LP site before fertilizer application are approximately 9.0, 6.8, and 1.6 t ha−1, respectively (Supplement Table S1, Lang et al. 2017). The pH of the soils ranged from 3.5 to 3.8 (Supplement Table S1). Information on the climate (1981 to 2010) and weather during sampling was obtained from www.wetterzentrale.de (Supplement Table 2).

For this study, 12 plots with an area of 400 m^2 each were installed in each forest in summer 2016 under 120- to 140-year-old beech trees (Supplement Table S1). One control (Con) and three different fertilizer treatments (N, P, P + N), each replicated three times per forest (= a total of 36 plots) and located about 300 m apart from each other, were treated as follows: P was applied only once in late summer 2016 as KH_2PO_4 at the dosage of 50 kg P ha^{-1} to the P and N + P plots. N was applied as NH_4NO_3 five times (late summer 2016, spring 2017, summer 2017, fall 2017, spring 2018) corresponding to a dosage of 30 kg N ha^{-1} per treatment on the N and N + P plots. To account for the K input in the P treatments, KCl was applied once in fall 2016 to the Con and N plots. The minerals were dissolved in tap water and applied with garden sprayers.

Harvest and processing of soil cores

Soil was sampled in the third year after the start of the treatments in spring (LP: 16.04.2018, HP: 23.04.2018, MP: 02.05.2018) and fall (LP: 17.09.2018, HP: 25.09.2018, MP: 01.10.2018). The weather conditions in the months of sampling and before as well as the long-term climate (1981 to 2010) are shown in the supplementary materials (Supplement Table S2). The sampling took place approximately 6 months after N addition. In each plot, 12 randomly distributed soil cores (depth 0.21 m, diameter 55 mm) were extracted after removal of surface litter. Each soil core was separated in organic (OL) and mineral topsoil (ML). The respective layers were pooled yielding one OL and one ML sample per plot. Each sample was fractionated into bulk soil, fine roots (< 2 mm), coarse roots (> 2 mm), and residual materials (fruits, twigs, and stones) in the field. Each subsample was directly divided into three aliquots: a fresh sample that was kept cool at 4 °C until use, a sample that was immediately frozen in liquid N (and stored at − 80 °C in the laboratory) and a sample that was dried (40 °C, 14 days). Bulk soil was sieved (mesh width: 4 mm) and the root samples were carefully washed before the aliquots were taken. All fractions were weighed in the laboratory. During the harvest in fall 2018, an additional soil core was collected in each plot. The sampling position was located adjacent to that of the soil cores for chemical analysis. The extra soil core was used to collect the roots for mycorrhizal morphotyping.

Determination of soil and root chemistry

To determine inorganic N, the concentrations of exchangeable ammonium (NH_4^+) and nitrate (NO_3^-) in soil, 20 g of fresh sieved bulk soil was extracted at the field site in 40 ml $CaCl_2$ solution for 60 min under shaking, subsequently filtered with phosphate free filter paper (MN 280 ¼, Macherey–Nagel, Düren, Germany) and kept cool. In the laboratory, the extracts were dried twice by cryodessication for 72 h (BETA I, Christ, Osterode am Harz, Germany) and dissolved in 1.5 ml ultra-pure water. The concentrated extracts were used to determine nitrate (# 109,713, Merck, Darmstadt, Germany) and exchangeable ammonium concentrations with kits (# 100,683, Merck) according to the manufacturer's instructions. The extinction of the assays was measured in an UV–Vis spectrophotometer (Shimadzu 1601, Hannover, Germany) at 690 nm for NH_4^+, and 340 nm for NO_3^-.

To determine soil pH values, 10 g of oven dried milled soil was suspended in 25 ml deionized water and shaken for 1 h at 200 rpm. After sedimentation of the particles, the pH was measured by a pH meter (WTW, Weilheim, Germany). After addition of 0.01 M $CaCl_2$ (1:5 soil-to-solution ratio) and 16 h equilibration, the samples were measured again (ISO10390, 2005).

To determine element contents, dry soil and root samples were milled (Retsch MN 400, Haan, Germany) to a fine powder before determining element contents. For the determination of total P (P_{tot}), 50 mg of the powder was weighed and extracted in 25 ml of 65% HNO_3 at 160 °C for 12 h according to Heinrichs et al. (1986). For the determination of the labile P (P_{lab}) fraction about 100 mg of soil or root powder was extracted in 150 ml of Bray-1 solution (0.03 N NH_4F, 0.025 N HCl) for 60 min on a shaker at 180 rpm (Bray and Kurtz 1945). The extracts were

filtered using phosphate free filter paper (MN 280 ¼, Macherey–Nagel) and used for elemental analysis by inductively coupled plasma–optical emission spectroscopy (ICP-OES) (iCAP 7000 Series ICP–OES, Thermo Fisher Scientific, Dreieich, Germany). P was measured at the wavelength of 185.942 nm (axial) and calibrated with a series of concentrations by element standards (P: 0.1 mg l^{-1} to 20 mg l^{-1}) (Einzelstandards, Bernd Kraft, Duisburg, Germany). In addition to P, we also determined K, Ca, Mg, Mn, Fe, Al, and S in the P_{tot} extracts.

To determine C and N, subsamples of 2 to 12 mg of soil or 1.5 mg of root powder were weighed into tin capsules (size of 4 × 6 mm, IVA Analysentechnik, Meerbusch, Germany) using a microbalance (Model MC5, Sartorius, Goettingen, Germany) before determining C and N contents. The range of weights for the soil samples was necessary to avoid overflow of the measuring unit of the mass spectrometer since the C concentrations in the OL and ML and between sand of other soil types varied drastically. The amounts of C and N of the soil and plant samples were measured at the KOSI (Kompetenzzentrum Stabile Isotope, Göttingen. Germany) using an isotope mass spectrometer (Delta Plus, Finnigan MAT, Bremen, Germany) and acetanilide (10.36% N, 71.09% C) as the standard.

Determination of ectomycorrhizal fungal species by morphotyping and Sanger sequencing

Roots from the extra soil core collected in fall were separated according to OL and ML, and immediately processed after sampling. The beech roots were gently washed in 4 °C precooled tap water, spread in water in a glass dish, and categorized according to their visual appearance under a stereomicroscope (Leica M205 FA, Wetzlar, Germany) as vital ectomycorrhizal, vital non-mycorrhizal or dry. All root tips in each soil core were inspected and counted. Two soil cores did not contain any fine roots. Ectomycorrhizal colonization and root tip mortality were calculated as follows:

$$\text{Ecomycorrhizal colonization (\%)} = \frac{\text{(number of vital mycorrhizal root tips)} \times 100}{\text{(number of vital mycorrhizal root tips + number of vital nonmycorrhizal root tips}}$$

$$\text{Root tip mortality (\%)} = \frac{\text{number of dry root tips}}{\text{number of all counted root tips}} \times 100$$

The abundance of different morphotypes was determined under a stereomicroscope (Leica M205 FA, Wetzlar, Germany) using a simplified identification key (after Agerer 1987–2012). All root tips in each soil core were categorized and counted. For mycorrhizal species identification, all different morphotypes were collected, which comprised at least three root tips per sample. Samples with no ectomycorrhizal root tips were included as zero values. We distinguished 44 morphotypes and sequenced 19 morphotypes, which were most abundant and covered > 90% of the beech root ectomycorrhizal fungal community. We used the protocol of Pena et al. (2017) for DNA extraction, ITS sequencing, and species identification. We used the primers ITS1F (5′CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). Fungal sequences have been deposited in NCBI GenBank under the accession numbers MT859114 to MT859131 (Supplement Table S3). Relative abundance of EMF species was calculated as follows:

$$\text{Relative abundance of EMF species (\%)} = \frac{\text{(number of root tips colonized by a EMF species)} \times 100}{\text{number of all vital ectomycorrhizal root tips}}$$

DNA extraction and preparation of soil and root samples for Ilumina sequencing

Frozen soil and root samples that had been stored at − 80 °C were milled in a ball mill (Retsch GmbH, Haan, Germany) in liquid N_2. DNA was isolated from 250 mg soil or from 180 mg roots with the DNeasy® PowerSoil® Pro kit (Qiagen, Hilden, Germany) or innuPREP Plant DNA kit (Analytik Jena AG, Jena, Germany), following the manufacturer's recommendations. DNA was purified using the DNeasy® PowerClean® Pro Cleanup kit (Qiagen). The amount of isolated DNA was measured in a NanoDrop ND-1000 spectrophotometer (PEQLAB Biotechnologie GmbH, Erlangen, Germany). For each DNA extraction, a PCR was performed in a reaction volume of 50 µl using 0.3 µl of Phusion High-Fidelity DNA Polymerase (2 U μl^{-1}, New England Biolabs (NEB), Frankfurt, Germany), 6 µl of 5 × Phusion HF buffer (NEB), 0.09 µl of $MgCl_2$ (50 mM, NEB), 0.6 µl of dNTP mix (10 mM each, Thermo Fisher Scientific, Osterode am Harz, Germany), 0.6 µl of the forward (ITS3-KYO2) and reverse primer (ITS4) (10 mmol/l, Microsynth, Wolfurt, Austria), and about 250 (roots) to 1050 (soil) ng of template DNA in 5 µl. The primers ITS3-KYO2 (Toju et al. 2012) and ITS4 (White et al. 1990) included the adapters for MiSeq sequencing. The PCR reactions were performed in a Labcycler (SensoQuest, Göttingen, Germany). The cycling

parameters were 1 cycle of 98 °C for 30 s; 30 cycles of 98 °C for 10 s, 47 °C for 20 s, and 72 °C for 20 s; and a final extension at 72 °C for 5 min. The PCR products were subjected to electrophoresis in 2% agarose gels (Biozym LE Agarose, Biozym Scientific GmbH, Hessisch Oldendorf, Germany) using GelRed (10 000 ×, VWR, Darmstadt, Germany) to stain the 1 kb DNA ladder (NEB) that was used for the determination of the product size. The PCR products were visualized with an FLA-5100 Fluorescence Laser Scanner (Raytest GmbH, Straubenhardt, Germany) and an Aida Image Analyzer v. 4.27 (Raytest GmbH). All PCR reactions were performed in triplicate, pooled, and purified using the MagSi-NGS^{PREP} Plus Kit (Steinbrenner Laborsysteme, Wiesenbach, Germany). Quantification of the purified PCR products was performed with a Quant-iT dsDNA HS assay kit (Life Technologies GmbH, Darmstadt, Germany) in a Qubit fluorimeter (Life Technologies GmbH, Darmstadt, Germany) following the manufacturer's recommendations.

Amplicon sequencing and bioinformatic processing

Amplicon sequencing was conducted at Göttingen Genomics Laboratory on the MiSeq platform using the MiSeq Reagent Kit v3 (Illumina Inc., San Diego, USA). For amplicon sequence variant (ASV) assembly paired-end sequencing data from the Illumina MiSeq were quality-filtered with fastp (version 0.20.0) using default settings with the addition of an increased per base phred score of 20, base pair corrections by overlap (-c), as well as 5′- and 3′-end read trimming with a sliding window of 4, a mean quality of 20 and minimum sequence size of 50 bp (von Hoyningen-Huene et al. 2019). Subsequently, quality-filtered reads were merged using PEAR v.0.9.11 (Zhang et al. 2014) with default parameters. Primer sequences were clipped with cutadapt v.2.5 (Martin 2011). VSEARCH v.2.14.1 (Rognes et al. 2016) was used for size exclusion of reads < 140 bp, dereplication, denoising (UNOISE3, default settings), and chimera removal (de novo followed by reference-based chimera removal). ASVs were clustered at 97% sequence identity [corresponding to operational taxonomic units (OTUs), the usual threshold in most fungal studies] employing VSEARCH (–sortbysize and –cluster_size). Reads were mapped to OTUs and used to create a count table using VSEARCH (–usearch_global, -id 0.97).

OTUs were taxonomically classified using the BLAST algorithm against the UNITE + INSDC 8.2 public database (Kõljalg et al. 2013) with an identity cutoff of 90%. Unclassified and non-blast hit OTUs (< 90% identity) were aligned against the GenBank (nt, 2020–01-17) database (Geer et al. 2010) and only OTUs with a fungal classification were kept in the OTU table. The OTU count table was rarefied to the count number of 11,000 (minimum number reads in one sample) using the rrarefy() function of the package vegan v2.5.6 (Oksanen et al. 2019). DESeq2 and Bonferroni correction of p values were used to test for significant differences between counts of distinct OTUs after fertilizer treatment and controls (Love et al. 2014). OTUs were functionally annotated as symbiotroph, pathotroph, and saprotroph using the FUNGuild database (Nguyen et al. 2016).

Statistical procedures and calculations

The statistical analyses were performed with R version 3.6.0 (R Core Development Team 2012). Normal distribution and homogeneity of variances were tested by analyzing the residuals of the models and performing a Shapiro–Wilk test for chemical soil and root parameters and EMF relative abundance. Data were logarithmically or square root-transformed to meet the criteria of normal distribution and homogeneity of variances, where necessary.

To determine the effects of forest type, soil layer, season, habitat, and treatment linear mixed effect models ("lmer," R package lme4) were used. The factor plot was used as random effect and the factor season (spring and fall) was defined as repeated measurement in the model. Pairwise comparisons of the sample means were conducted using Tukey's HSD (package: "multcomp"). Means were considered to be significantly different from each other when $p \leq 0.05$. Data are shown as means and standard error (± SE) of the three plots per treatment, if not indicated otherwise. If not indicated otherwise, count data were not transformed. Visual inspection of their residuals showed homogenous distribution and therefore, these data also were analyzed by linear mixed effect models using a quasi-Poisson distribution. P values of repeated tests were adjusted with Bonferroni, as indicated in figure and table legends. Shannon diversity and fungal richness were determined with the package "vegan" (Oksanen et al. 2019) and analyzed by generalized linear models. For comparisons of OL and ML a paired rank test was used.

We used the following fungal communities for our analyses: EMF, SAF, and RAF. RAF and SAF were distinguished in the Illumina dataset and further discerned as SYM, SAP, and PAT corresponding to the groups of ectomycorrhizal (SYM), saprotrophic (SAP), and pathogenic fungi (PAT). To obtain ectomycorrhizal fungi, the guild of symbiotrophic fungi was manually screened and other symbiotrophic fungi (arbuscular mycorrhizal, orchid mycorrhizal, endophyte, lichenized, combinations of saprotrophic, or pathotrophic with EMF) were eliminated and then used as SYM for further analyses.

Detrended correspondence analysis (DCA) was used to explore and visualize fungal community composition. The function ADONIS (multivariate analysis of variance using distance matrices) (package: "vegan") (Oksanen et al. 2019) was used to analyze the dissimilarities among the fungal

communities for the factors: forest type, habitat, layer, season, and treatment.

To determine the influence of fertilization in different soil layers and seasons, we grouped fungal taxa according to fungal orders. We used the most abundant fungal orders, which encompassed > 1% of the fungal sequences to calculate Generalized Adaptive Models for Location, Scale and Shape with a zero-inflated beta family (GAMLSS-BEZI) with the R package "metamicrobiomeR" (Ho et al. 2019). By using a zero-inflated beta (BEZI) family, GAMLSS regression model is applicable for any distribution type exhibited by a response variable (Rigby and Stasinopoulos, 2005). GAMLSS can be used for analyses of relative abundance data and utilizes the log of odds ratio to compute meta-analysis (Ho et al., 2019).

Results

Influence of P and N addition on soil and root chemistry

Soil and root chemistry varied with forest site and season, while treatment effects due to P, N, or N + P addition were mainly found for P (Table 1). P addition resulted in increased soil P_{lab} concentrations across the three study forests but did not affect the soil P_{tot} concentrations (Fig. 1a-c, Table 1). The effects were also present when the forests were fertilized with P + N (Fig. 1a–c) and were more pronounced during fall than spring (Table 1, Supplement Table S4). Furthermore, the P fertilization effects were stronger in the organic layer than in the mineral soil (Fig. 1a–c, Table 1, HP: $F = 12.6$, $p = 0.001$; LP: $F = 111.1$, $p < 0.001$, lmer), with the exception of the MP forest soils ($F = 0.02$, $p = 0.902$, lmer, Fig. 1b).

In P-fertilized forest soils with higher availabilities of P_{lab}, the fine root P_{tot} concentrations were only higher in the LP forest, whereas P_{tot} in fine roots in the HP and MP forests were unaffected compared with the controls (Fig. 2a–c). In all three forests, the P_{lab} concentrations of the roots were higher after P fertilization (Fig. 2d–f). Across the three forests, fine root P_{lab} was higher during spring (556 ± 29 µg g^{-1} dw) than fall (320 ± 19 µg g^{-1} dw, $F = 25.3$, $p < 0.001$) and higher in the organic layer (526 ± 31 µg g^{-1} dw) than in the mineral layer (351 ± 20 µg g^{-1} dw, $F = 22.0$, $p < 0.001$, lmer) (Supplement Table S4).

N fertilization slightly lowered the C/N ratio in the MP forest and raised it in the LP forest (Table 1, Supplement Table S4). The N addition did not increase soluble, inorganic N concentrations (NO_3^-, exchangeable NH_4^+) in the mineral soil (Fig. 1d–f). In the organic layer, the N-fertilized MP forest contained lower exchangeable NH_4^+ concentrations than the controls (Fig. 1e), while no effect was found in the HP and an increase in the LP forest (Fig. 1d,f). No significant seasonal effects of the treatments on the NO_3^- or exchangeable NH_4^+ concentrations were observed (Table 1).

N fertilization significantly decreased root P_{tot} concentrations in the organic layer (Fig. 2a–c) but did not change root N concentrations (Table 1, Supplement Table S4). Therefore, the treatments increased the N:P ratios of the roots but not of the soil (Table 1, Supplement Table S4).

Influence of P and N addition on EMF and root- or soil-associated fungal diversity indices and fungal community composition

We analyzed fungal species richness and Shannon diversity at three scales: EMF, RAF, and SAF (Fig. 3, Table 2). EMF richness did not vary in response to the treatments (Fig. 3a, Table 2). We detected a mean of 8.0 ± 0.4 EMF species per treatment and forest. The dominant EMF species belonged to the genera Russula, Lactarius, Xerocomus, Laccaria, Hydnotrya, Elaphomyces, Clavulina, and Cenococcum and various members of Helotiales (Supplement Fig. S1). The Shannon diversity of EMF was unaffected by the fertilizer treatments (Fig. 3d, Table 2). Fertilization did not affect the fraction of mycorrhizal root tips (99.9 ± 0.4%) nor the fraction of dead root tips (29.7 ± 1.6%) (Supplement Table S5). However, in the mineral layer in the MP and LP forests, root mortality was higher than that in the HP forest (Supplement Table S5).

Using Illumina sequencing (288 samples in total), we obtained 3169 million fungal sequence reads, which clustered into 4134 different OTUs. Across all conditions, the RAF communities contained approximately three times fewer OTUs (219 ± 12) than the SAF communities (764 ± 10, $F = 16{,}155$, $p < 0.001$). P fertilization caused a reduction in the OTU richness of RAF in the organic layer (Fig. 3b) but did not affect Shannon diversity (Fig. 3e). The N and N + P treatments did not have any effects on RAF richness or Shannon diversity (Fig. 3b, e, Table 2). SAF richness and Shannon diversity were unaffected by any fertilizer treatment (Fig. 3c, f, Table 2).

Variations in RAF and SAF OTU richness or Shannon diversity were often observed during different seasons and among the forests (Table 2). OTU richness in the organic layer of RAF and SAF was higher in the organic layer than in the mineral soil (Fig. b,c).

Differential abundance measurements aimed at identifying the distinct fungal OTUs that responded to treatments were unsuccessful because the majority of the OTUs were represented by low and variable numbers of sequence reads spread across 92 fungal orders (Supplement Table S6). We tested whether fertilizer treatments influenced fungal community composition, but we did not detect any treatment effects, whereas forest, soil layer and season caused

Table.1 Statistical information on the effects of forest site, season and fertilization treatments on soil and root chemistry in the organic layer and in the mineral topsoil. Soils and roots were collected in a P-rich (HP), P-medium (MP), and P-poor (LP) forest in spring and fall 2018 and separated into organic layer and the mineral topsoil for analyses. Means can be found in the supplementary information (Supplement Table S4). Differences among means per forest type, season, treatment, and the two-way interactions (forest × season, forest × treatment, season × treatment, P × N) were tested by a linear mixed effect model with plot number as random effect. Calculations were performed separately for the organic layer and mineral topsoil. Tukey HSD was used as posthoc test. Bold letters indicate significant differences at $p \leq 0.05$. RWC = relative water content. $p = p$-value

	Forest		Season		Treatment		Forest × season		Forest × treatment		Season × treatment		N		P		P × N	
	F	*P*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*P*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Soil: Organic layer																		
RWC	77.5	**< 0.001**	221.8	**< 0.001**	0.9	0.451	37.4	**< 0.001**	0.9	0.502	2.4	0.078	0.0	0.989	0.3	0.608	0.1	0.705
P_{tot}	315.5	**< 0.001**	73.8	**< 0.001**	1.6	0.200	4.0	**0.025**	4.0	**0.003**	2.3	0.085	0.8	0.379	0.5	0.475	0.1	0.722
P_{lab}	120.8	**< 0.001**	65.8	**< 0.001**	7.7	**< 0.001**	4.2	**0.020**	0.7	0.651	2.3	0.089	0.0	0.980	10.4	**0.002**	0.0	0.879
N	57.7	**< 0.001**	21.5	**< 0.001**	1.6	0.212	1.9	0.154	0.2	0.976	1.8	0.163	0.0	0.879	2.2	0.142	0.6	0.448
C	52.6	**< 0.001**	42.6	**< 0.001**	1.1	0.346	3.2	**0.048**	0.1	0.993	2.0	0.134	0.0	0.879	2.2	0.142	0.6	0.431
C:N	53.1	**< 0.001**	51.9	**< 0.001**	1.3	0.283	20.3	**< 0.001**	1.2	0.208	0.4	0.717	0.4	0.493	0.5	0.470	0.0	0.886
N:P	123.9	**< 0.001**	11.9	**0.001**	0.5	0.674	3.4	**0.042**	0.4	0.888	0.5	0.665	0.0	0.974	0.0	0.923	0.4	0.531
pH	148.2	**< 0.001**	151.2	**< 0.001**	2.6	0.066	15.7	**< 0.001**	0.8	0.546	0.6	0.588	1.8	0.180	0.1	0.723	0.0	0.947
NH_4^+	6.9	**0.002**	2.6	0.112	0.9	0.459	1.8	0.172	2.9	**0.018**	0.6	0.590	2.0	0.166	0.6	0.449	0.1	0.785
NO_{3-}^-	11.0	**< 0.001**	3.9	0.054	0.2	0.910	1.3	0.272	0.3	0.952	1.1	0.340	0.4	0.508	0.0	0.997	0.1	0.824
Soil: Mineral layer																		
RWC	324.6	**< 0.001**	206.5	**< 0.001**	3.5	**0.022**	0.7	0.482	2.0	0.088	4.1	**0.012**	0.8	0.388	1.2	0.280	0.1	0.744
P_{tot}	1363.0	**< 0.001**	270.1	**< 0.001**	3.2	**0.031**	107.4	**< 0.001**	2.1	0.065	0.1	0.951	0.8	0.365	0.2	0.644	0.0	0.948
P_{lab}	105.3	**< 0.001**	15.5	**< 0.001**	1.5	0.218	7.4	**0.002**	2.4	**0.044**	1.3	0.301	0.0	0.917	3.2	0.081	0.0	0.915
N	291.1	**< 0.001**	0.1	0.815	2.6	0.067	0.2	0.815	2.0	0.078	1.9	0.136	4.7	**0.034**	4.6	**0.037**	0.2	0.627
C	135.5	**< 0.001**	0.2	0.665	1.4	0.268	0.5	0.630	2.1	0.070	1.7	0.188	1.9	0.176	4.2	**0.045**	0.4	0.516
C:N	815.7	**< 0.001**	41.1	**< 0.001**	5.5	**0.002**	73.5	**< 0.001**	8.1	**< 0.001**	7.5	**< 0.001**	0.0	0.875	0.5	0.467	0.0	0.924
N:P	59.3	**< 0.001**	1.4	0.248	0.3	0.849	0.6	0.569	0.4	0.864	0.2	0.871	0.2	0.665	0.1	0.821	0.1	0.838
pH	58.3	**< 0.001**	8.1	**0.007**	0.5	0.692	4.0	0.024	1.2	0.312	0.3	0.810	0.4	0.554	0.4	0.546	0.1	0.718
NH_4^+	1.7	0.195	1.6	0.216	0.5	0.664	0.1	0.951	1.3	0.259	0.6	0.606	0.0	0.872	1.0	0.332	1.2	0.282
NO_3^-	1.9	0.168	2.8	0.102	0.3	0.790	4.8	0.012	1.0	0.414	1.3	0.280	0.4	0.511	0.5	0.503	0.0	0.902

Table.1 (continued)

	Forest		Season		Treatment		Forest × season		Forest × treatment		Season × treatment		N		P		P × N	
	F	*P*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*P*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Fine roots: Organic layer																		
RWC	9.7	**< 0.001**	101.4	**< 0.001**	0.5	0.678	6.8	0.002	0.4	0.904	0.4	0.783	0.0	0.891	0.4	0.538	0.1	0.707
P_{tot}	86.2	**< 0.001**	188.7	**< 0.001**	8.8	**< 0.001**	12.6	**< 0.001**	1.8	0.111	0.4	0.776	3.8	0.057	0.8	0.386	0.8	0.373
P_{lab}	3.0	0.057	69.1	**< 0.001**	6.6	**0.001**	10.1	**< 0.001**	1.0	0.441	1.6	0.209	0.1	0.755	7.8	**0.007**	0.0	0.902
N	11.4	**< 0.001**	3.0	0.089	0.5	0.679	29.3	**< 0.001**	0.4	0.878	0.3	0.856	0.1	0.771	0.3	0.579	0.4	0.535
C	119.5	**< 0.001**	0.1	0.733	0.5	0.714	17.9	**< 0.001**	0.7	0.660	0.1	0.944	0.0	0.866	0.8	0.361	0.0	0.925
C:N	19.4	**< 0.001**	4.1	**0.048**	0.6	0.572	21.3	**< 0.001**	0.2	0.965	0.5	0.642	0.0	0.826	0.3	0.587	0.6	0.423
N:P	30.1	**< 0.001**	98.3	**< 0.001**	3.9	**0.013**	19.3	**< 0.001**	0.5	0.808	0.2	0.886	1.5	0.227	2.3	0.134	0.1	0.675
Fine roots: Mineral layer																		
RWC	71.1	**< 0.001**	1.5	0.231	2.1	0.111	34.7	**< 0.001**	0.5	0.803	1.1	0.376	0.9	0.352	0.5	0.471	0.1	0.706
P_{tot}	110.4	**< 0.001**	10.2	**0.003**	4.5	**0.007**	0.2	0.802	2.7	**0.025**	2.3	0.093	5.9	**0.018**	4.4	**0.040**	0.8	0.373
P_{lab}	94.4	**< 0.001**	69.1	**< 0.001**	12.2	**< 0.001**	3.8	**0.028**	1.7	0.149	2.3	0.093	1.1	0.291	14.2	**< 0.001**	0.0	0.899
N	58.2	**< 0.001**	3.4	0.073	2.2	0.105	15.1	**< 0.001**	0.4	0.880	1.2	0.338	3.6	0.064	0.2	0.620	0.4	0.545
C	9.2	**< 0.001**	6.4	**0.015**	0.6	0.587	4.1	**0.022**	0.6	0.708	0.1	0.952	1.4	0.241	0.2	0.692	0.0	0.925
C:N	65.1	**< 0.001**	2.0	0.164	3.3	**0.028**	9.9	**< 0.001**	1.1	0.401	0.8	0.467	2.5	0.116	0.2	0.691	0.6	0.423
N:P	47.9	**< 0.001**	13.6	**< 0.001**	7.0	**< 0.001**	7.3	**0.002**	3.1	**0.011**	5.3	**0.003**	1.7	0.195	4.1	**0.045**	0.2	0.675

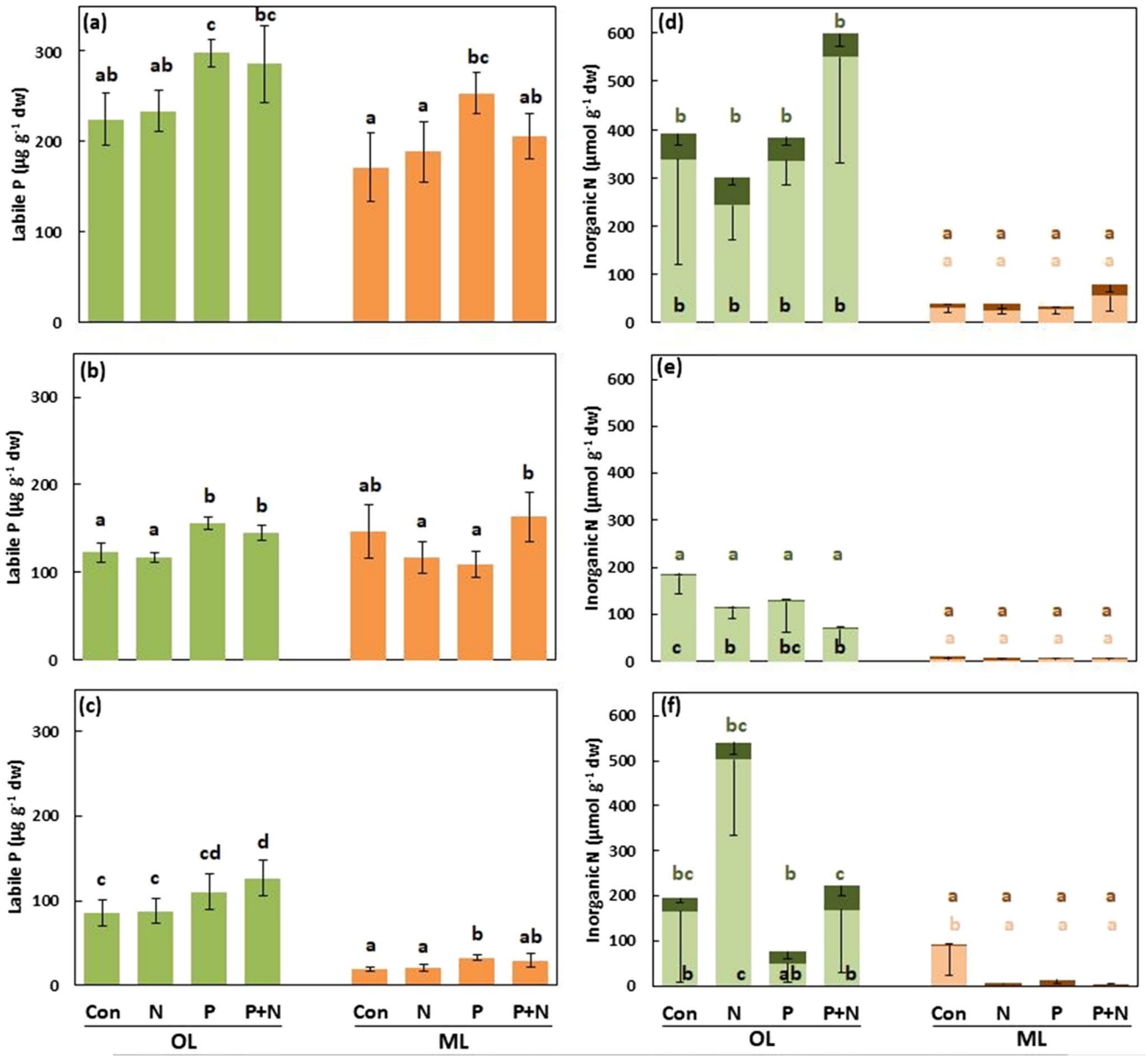

Fig. 1 Labile phosphorus (**a**, **b**, **c**) and inorganic nitrogen (**d, e**, **f**) concentrations the soil of beech forests (*Fagus sylvatica* L.) Soils were collected in a P-rich (**a**, **d**), P-medium (**b**, **e**), and P-poor (**c**, **f**) forest and separated into organic layer (green) and the mineral topsoil (orange) for analyses. Stacked bars show ammonium (bright colors) and nitrate (dark colors). Data for spring and fall were pooled. Data indicate means ($n = 6 \pm$ SE). Differences among means were tested by a linear mixed effect model with plot number as random effect and Tukey HSD post hoc test. Different letters indicate significant differences at $p \leq 0.05$

significant differentiation within the EMF, RAF, and SAF communities (Supplement Fig. S2a, b, c).

Phylogenetic and functional groups of fungi respond to N and P treatments

Since important fungal traits for nutrient use and turnover are conserved at higher classification levels (Treseder and Lennon, 2015), we assigned the OTUs to fungal orders (Supplement Table S6). Fifteen of the 92 detected orders were abundant, each accounting for more than 1% of the fungal sequences (Supplement Fig. S3, Supplement Table S6). Seven orders were unaffected by the fertilizer treatments (Agaricales, Atheliales, Cantharellales, Thelephorales, Eurotiales, Hypocreales, and Mortierellales, Supplement Figs. S4 and S5).

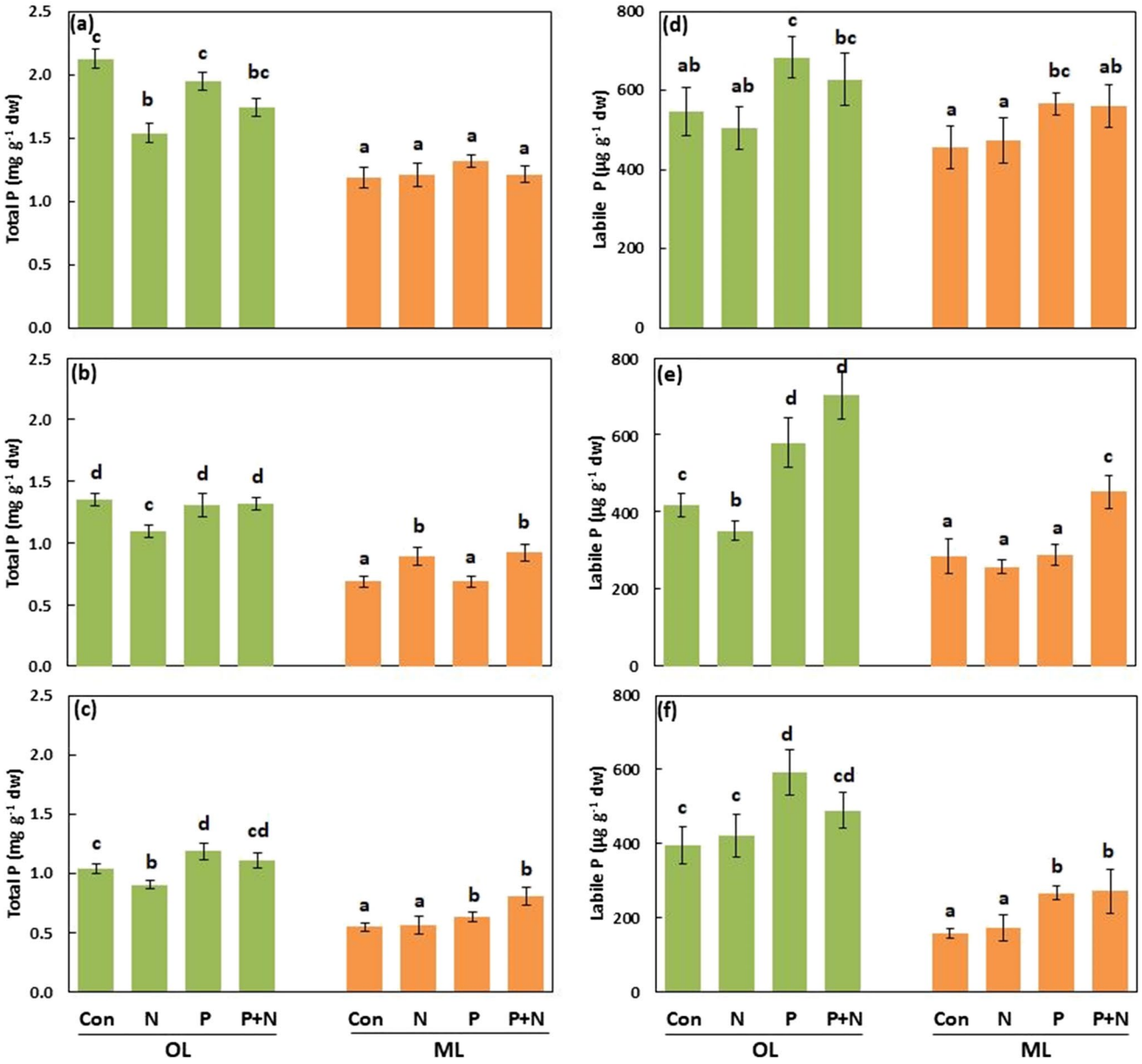

Fig. 2 Total phosphorus (**a**, **b**, **c**) and labile phosphorus (**d**, **e**, **f**) in roots of beech forests (*Fagus sylvatica* L.) Fine roots were collected in a P-rich (**a**, **d**), P-medium (**b**, **e**), and P-poor (**c**, **f**) forest and separated into organic layer (green) and the mineral topsoil (orange) for analyses. Season data were pooled. Data indicate means ($n = 6 \pm$ SE). Differences among means were tested by a linear mixed effect model with plot number as random effect and Tukey HSD post hoc test. Different letters indicate significant differences at $p \leq 0.05$

A heatmap differentiating the organic and mineral soil fungal responses during spring and fall showed that four RAF orders (Russulales, Boletales, Trechisporales, and Pezizales) and seven SAF orders (Boletales, Trechisporales, Helotiales, Hypocreales, Sodariales, Pleosporales, and Mytilinidales) were significantly affected by the fertilization treatments (Fig. 4). Only SAF responded to N, with negative responses for Helotiales in spring in the mineral soil and positive responses for the Hypocreales in the organic layer when both seasons were considered together (Fig. 4). Further positive effects on the SAF were observed after P or N + P treatment, but the responses of different orders were scattered among different soil layers and seasons (Fig. 4). When the SAF data for seasons and soil layers were analyzed together, the treatment effects were masked (Fig. 5a).

Among the RAF, Russulales and Boletales showed the most consistent and strongest responses to the P and N + P treatments (Fig. 4). The P treatment caused relative enrichment of Russulales during spring in both soil layers and a decrease during fall compared with that in the controls

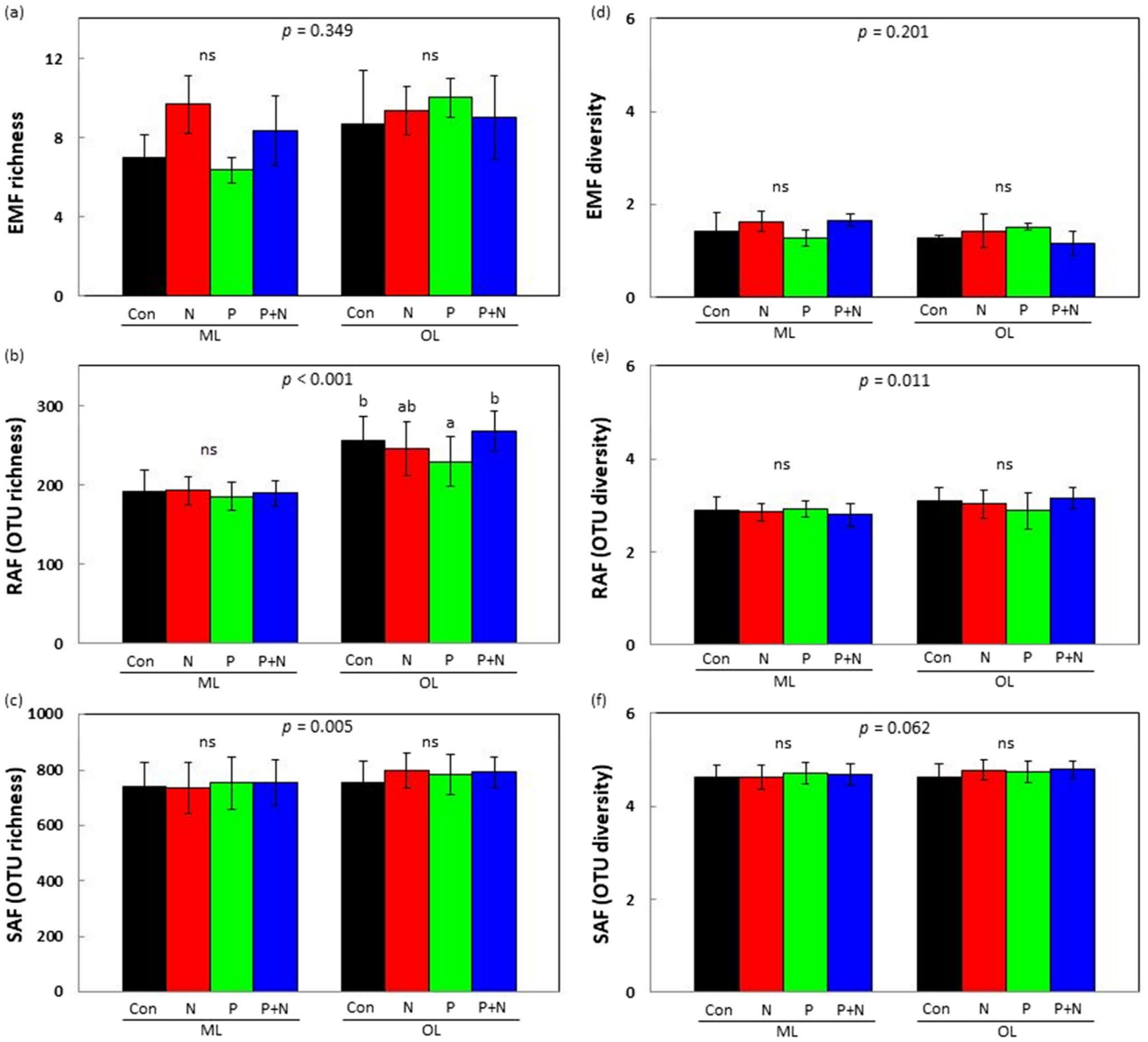

Fig. 3 Richness (**a**, **b**, **c**) and Shannon diversity (**d**, **e**, **f**) of ectomycorrhizal (EMF), root (RAF), and soil (SAF) associated fungi in the organic layer (OL) and in the mineral topsoil (ML) in response to fertilization (Con, N, P, P + N). Soils and fine root samples were collected in a P-rich, P-medium, and P-poor beech forest (*Fagus sylvatica* L.) in spring and fall 2018 and separated into organic layer and the mineral topsoil for analyses. Data indicate means ($n = 36 \pm$ SE). Differences among treatments were tested by generalized linear models followed by a post hoc test (Tukey). Calculations were performed separately for the organic layer and mineral topsoil. Data for richness were log transformed prior analyses. Different letters indicate significant differences at $p \leq 0.05$ for each soil layer seperatley. Controls (Con) = black, N = red, P = green, P + N = blue. ns., not significant. ***P*** values in each subpanel refer to the comparisons between ML and OL conducted by a paired rank test. Further statistical information is shown in Table 2

(Fig. 4). The Boletales in the RAF showed higher relative abundances in response to P and N + P fertilization during fall compared with those in the controls (Fig. 4). When the data for RAF in different soil layers and seasons were pooled, the P treatment still resulted in approximately twice as high relative abundances of Boletales than those in the controls (Fig. 5b), whereas the positive effects of P on Russulales during spring were masked by negative effects during fall (Fig. 5b). N + P treatment significantly reduced Russulales in the RAF (Fig. 5b). Other fungal orders in the RAF that responded to fertilization were Trechisporales, which showed positive effects under the N + P treatment in the mineral layer, and Pezizales, which showed negative effects under the P treatments in the organic layer during spring (Fig. 4).

Table.2 Statistical information on the effects of forest site, season and fertilization treatments on richness and Shannon diversity of soil- and root-associated fungi and on ectomycorrhizal fungi colonizing the root tips in the organic layer and in the mineral topsoil. Soil and fine root samples were collected in a P-rich, P-medium, and P-poor forest in spring and fall 2018 and separated into organic layer and the mineral topsoil for analyses. Differences among means per forest type, season, and treatment were tested by a generalized linear model (GLM) with lg-transformed data. Calculations were performed separately for the organic layer and mineral topsoil. Interactions were tested but not found. Bold letters indicate significant differences at $p \leq 0.05$. SoS = sum of squares, MS = mean square, R^2 (ad) indicates the explained variance adjusted for degrees of freedom

		Organic layer				Mineral soil			
Richness	Soil-associated fungi								
Source	Df	SoS	MS	*F*-ratio	*P* value	SoS	MS	*F*-ratio	*P* value
Model	6	0.1905	0.0318	16.97	**< 0.0001**	0.4927	0.0821	17.81	**< 0.0001**
Treatment	3	0.0067	0.0022	1.19	0.3220	0.0021	0.0007	0.15	0.9276
Forest	2	0.0129	0.0065	3.45	**0.0377**	0.2656	0.1328	28.79	**< 0.0001**
Season	1	0.1710	0.1710	91.39	**< 0.0001**	0.2250	0.2250	48.80	**< 0.0001**
Residual	65	0.1216	0.0019			0.2997	0.0046		
Total (corrected)	71	0.3121			R^2 (adj) = 57.4%	0.7925			R^2 (adj) = 58.7%
Shannon diversity									
Source	Df	SoS	MS	*F*-ratio	*P* value	SoS	MS	*F*-ratio	*P* value
Model	6	4.8024	0.8004	9.03	**< 0.0001**	6.0542	1.0090	10.06	**< 0.0001**
Treatment	3	0.2751	0.0917	1.04	0.3830	0.0692	0.0231	0.23	0.8751
Forest	2	0.1464	0.0732	0.83	0.4423	4.6718	2.3359	23.29	**< 0.0001**
Season	1	4.3809	4.3809	49.45	**< 0.0001**	1.3132	1.3132	13.10	**0.0006**
Residual	65	5.7585	0.0886			6.5184	0.1003		
Total (corr)	71	10.5609			R^2 (adj) = 40.4%	12.5726			R^2 (adj) = 43.4%
Richness	Root-associated fungi								
Source	Df	SoS	MS	*F*-ratio	*P* value	SoS	MS	*F*-ratio	*P* value
Model	6	0.3024	0.0504	10.03	**< 0.0001**	0.0254	0.0042	0.70	0.6506
Treatment	3	0.0537	0.0179	3.56	**0.0188**	0.0022	0.0007	0.12	0.9475
Forest	2	0.1138	0.0569	11.33	**0.0001**	0.0075	0.0037	0.62	0.5431
Season	1	0.1349	0.1349	26.85	**< 0.0001**	0.0158	0.0158	2.60	0.1114
Residual	65	0.3265	0.0050			0.3937	0.0061		
Total (corr)	71	0.6289			R^2 (adj) = 43.3%	0.4192			R^2 (adj) = 0.0%
Shannon diversity									
Source	Df	SoS	MS	*F*-ratio	*P* value	SoS	MS	*F*-ratio	*P* value
Model	6	5.9332	0.9889	4.87	**0.0004**	0.9767	0.1628	1.14	0.3516
Treatment	3	0.7756	0.2585	1.27	0.2911	0.1481	0.0494	0.34	0.7932
Forest	2	3.1337	1.5669	7.71	**0.0010**	0.4777	0.2388	1.67	0.1968
Season	1	2.0239	2.0239	9.96	**0.0024**	0.3509	0.3509	2.45	0.1224
Residual	65	13.2041	0.2031			9.3125	0.1433		
Total (corr)	71	19.1373			R^2 (adj) = 24.6%	10.2891			R^2 (adj) = 1.1%
Richness	Ectomycorrhizal fungi on root tips								
Source	Df	SoS	MS	*F*-ratio	*P* value	SoS	MS	*F*-ratio	*P* value
Model	5	34.4167	6.8833	1.69	0.2707	92.1667	18.4333	6.70	0.0192
Treatment	3	14.2500	4.7500	1.16	0.3984	2.0000	0.6667	0.24	0.8639
Forest	2	20.1667	10.0833	2.47	0.1650	90.1667	45.0833	16.39	**0.0037**
Residual	6	24.5000	4.0833			16.5	2.75		
Total (corr)	11	58.9167			R^2 (adj) = 23.8%	108.667			R^2 (adj) = 72.2%
Shannon diversity									
Source	Df	SoS	MS	*F*-ratio	*P* value	SoS	MS	*F*-ratio	*P* value
Model	5	0.4782	0.0956	0.58	0.7199	0.6851	0.1370	0.71	0.6386
Treatment	3	0.2354	0.0785	0.47	0.7131	0.2875	0.0958	0.50	0.6985
Forest	2	0.2428	0.1214	0.73	0.5203	0.3976	0.1988	1.03	0.4130
Residual	6	0.9980	0.1663			1.1597	0.1933		

Table.2 (continued)

		Organic layer		Mineral soil	
Total (corr)	11	1.4763	R^2 (adj) = 0.0%	1.8445	R^2 (adj) = 0.0%

Since the treatment effects on the RAF were confined to orders that consisted of EMF and in the SAF mainly to saprotrophic orders (except Boletales and Trechisporales), we tested whether N, P or P + N treatment (Fig. 5b) influenced the relative abundances of symbiotrophs or saprotrophs. The relative abundance of symbiotrophic fungi in the SAF and RAF increased, whereas that of saprotrophic fungi decreased in these compartments under the P and N treatments (Fig. 5c, d). We excluded pathotropic fungi from these analyses because their mean relative abundance was below 1%.

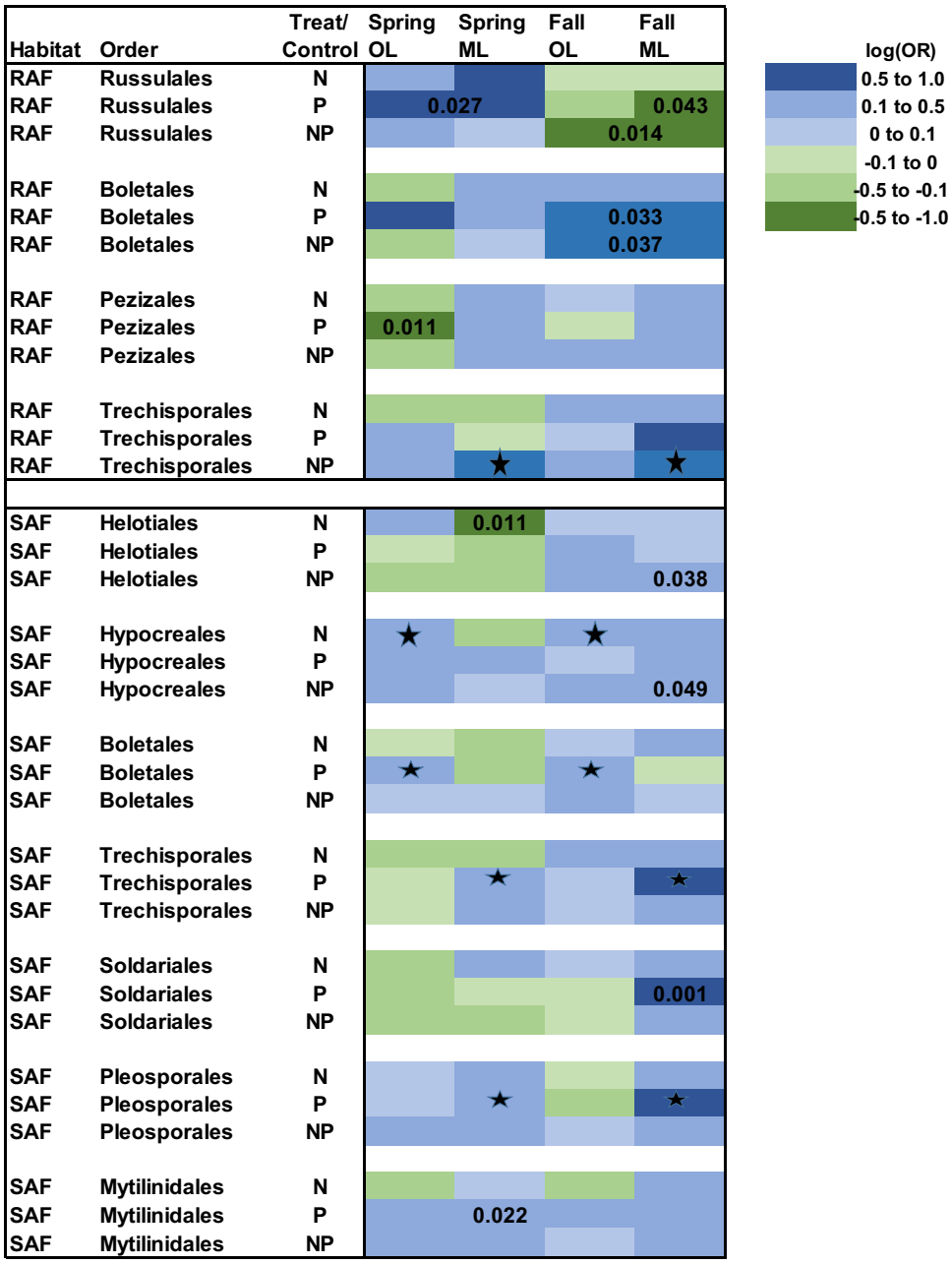

Fig. 4 Heatmap for the effect sizes of N, P, or N + P treatments on selected fungal orders relative to controls. RAF, root-associated fungi; SAF, soil-associated fungi; OL, organic layer; ML, mineral soil. Blue show positive and green color code negative responses. The responses are indicated as log of odds ratio (log(OR)) as the result of GAMLSS analyses. Numbers in cells indicate *p* values for significant changes. *P* values for shared effects of ML + OL are centered. Shared effects of spring + fall are indicated by * when $p \leq 0.05$

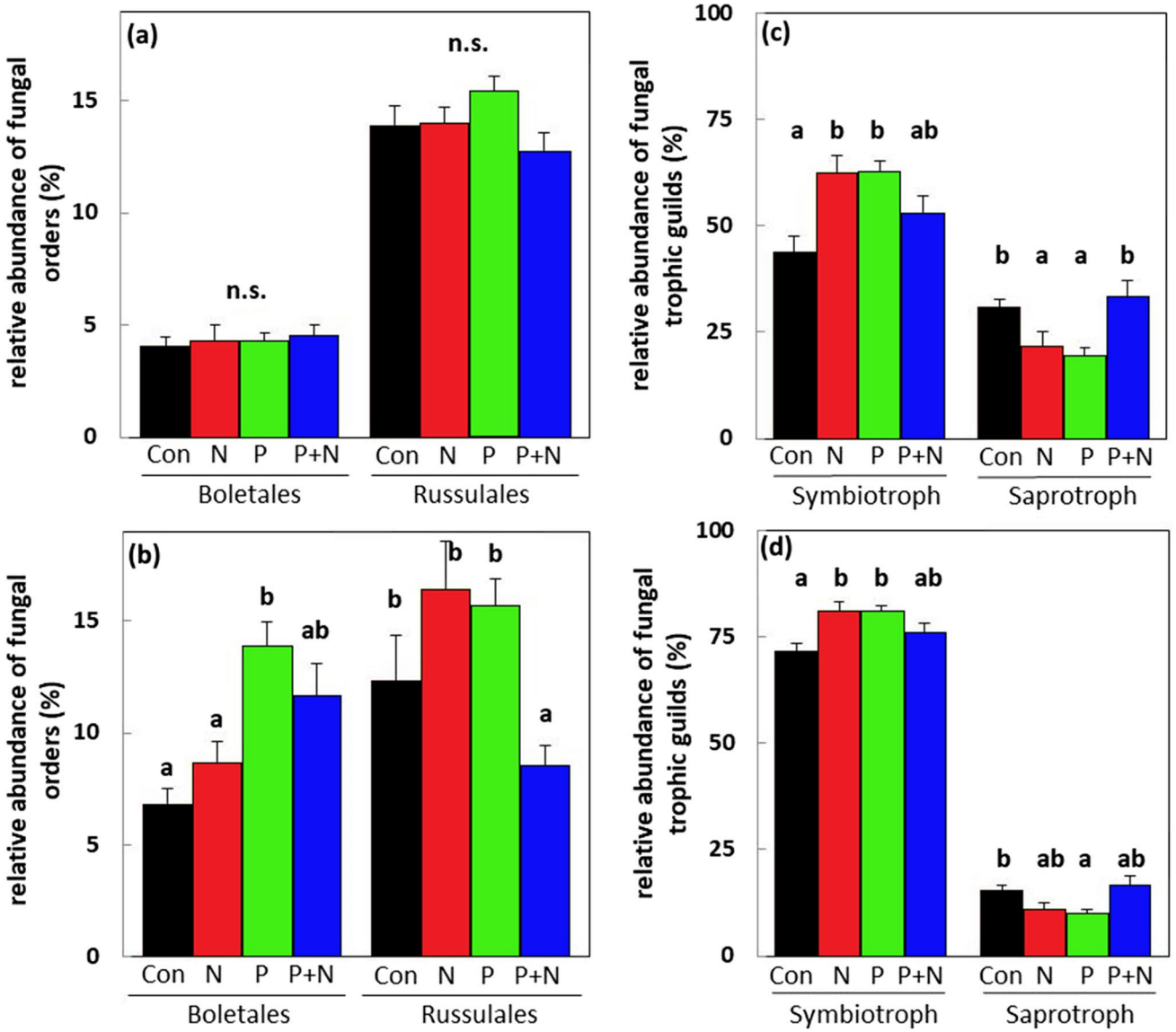

Fig. 5 Relative abundance of Boletales and Russulales in soil (**a**) and roots (**b**) and of trophic guilds in soil (**c**) and roots (**d**) in response to fertilization (Con, N, P, P + N). Soil and fine root samples of the organic layer and mineral topsoil were collected in a P-rich, P-medium, and P-poor beech forest (*Fagus sylvatica* L.) in spring and fall 2018. Data of the forest types and season were merged to evaluate effects of the treatments on the soil-residing fungi (**a**, **c**) and root-associated fungi (**b**, **d**). Data indicate means ($n = 36 \pm$ SE). Significant differences between the treatments were calculated by a linear mixed effect model using Poisson distribution and Tukey HSD post hoc test for the treatments with site as random effect and season as repeated measure. Different letters indicate significant differences for each fungal order and trophic group separately. Controls (Con) = black, N = red, P = green, P + N = blue. n.s., not significant

Discussion

P and N inputs affect P nutrition of beech

In agreement with our expectations, we found that under low P soil availabilities, root P concentrations decreased with N addition and increased with P addition. Unexpectedly, we found that N addition also decreased the P concentrations in roots in soils with higher P availabilities, i.e., in the HP and MP forests. These observations suggest that the applied amounts of N (here, 60 N kg ha^{-1} a^{-1}, other studies: 15 to 5 kg N $ha^{-1}a^{-1}$, De Vries et al. 2014; Gonzales and Yanei, 2019; Wardle et al. 2016), which exceed ambient deposition in unpolluted areas (approximately 6 kg N ha^{-1} a^{-1}, Schwede et al. 2018), might have caused N:P imbalances. Etzold et al. (2020) reported tipping points at 24 to 34 kg N ha^{-1} a^{-1} for positive

growth responses of forest trees in Europe, with potential negative effects at higher deposition values. In a meta-analysis, Deng et al. (2016) reported decreases in tissue P concentrations upon N fertilization, although the labile P pools in soils were unaffected. Our results are consistent with these findings.

Several previous studies in LP and HP forests clearly demonstrated that soil microbes and young beech trees in LP soil are limited by low P availabilities (Bergkemper et al. 2016; Lang et al. 2017; Pastore et al. 2020; Zavišić et al. 2018). Experimental studies with young trees in HP and LP soil showed that negative effects of P limitation, such as a reduction in photosynthesis were mitigated by P fertilization, while the photosynthesis of beech trees in HP soil was unaffected by P addition (Zavišić et al. 2018). In the present study, the NH_4^+ enrichment after N addition in LP, but not in HP soil suggests that N utilization was impaired due to P shortage. The observation that the accumulation of NH_4^+ in soil was relieved by P fertilization further supports this suggestion. N turnover is rapid, and great variations in NO_3^- and exchangeable NH_4^+ in soil solutions are known (Cheng et al. 2019; Ollivier et al. 2011). Although our analyses of NO_3^- and NH_4^+ represent only snapshots during our sampling campaigns, our results agree with those of other studies (Li et al. 2015; Liu et al. 2013; Xia et al. 2020), by showing that P addition counteracted the negative effects of high N input on root P contents.

P addition increased potential plant-available P in all three forest soils. This result might have been expected since the annual P uptake of forest trees is much lower (ca. 9 kg ha^{-1} a^{-1}, Rosling et al. 2016) than the amount of added P. In our study, the increase in P_{lab} was small compared to the content of P_{tot} in soil; therefore, we did not observe significant increases in P_{tot}. Consequently, the N:P ratios of the soils remained stable after N and P additions. Soil N:P ratios of approximately 15 have been suggested to indicate a nutritional balance in beech forests (Mellert and Göttlein 2012). Here, we found extremely large differences in these ratios among the forests and seasons (organic layer: 7 to 31, mineral layer: 3 to 17). Plant-available fractions of N and P are critical to tree nutrition. Here, the N_{min}:P_{lab} ratios decreased slightly in response to P or N addition in the HP forest but increased more than threefold after N addition in the organic layer of the LP forest compared to that in the HP forest (estimated with data from the organic layer, Supplement Table S4). This dynamic was also partly detected in roots, where N fertilization decreased P_{tot}, while P fertilization increased P_{lab}. These results support the metabolic flexibility of beech to cope with differences in nutrient availabilities (Meller et al. 2019; Zavišić et al., 2016).

Fungal taxonomic composition is driven by long-term habitat conditions

A main goal of this study was to disentangle the soil fungal community composition response to the addition of N, P, and P + N under conditions of P shortage or P sufficiency. In general, the assembly of soil fungi is predominately driven by deterministic processes, such as abiotic habitat conditions and soil properties, while stochastic effects play a minor role (Chase, 2007; Glassman et al. 2017; Mykrä et al. 2016; Peay et al. 2016; Štursová et al. 2014). In agreement with the expectation that abiotic habitat filters are important drivers of fungal community composition, our results showed that environmental variables such as humidity, P, Ca and N in soil and roots explained the variation in fungal community composition in different forests.

In line with the findings of other studies (Goldmann et al. 2016; Zhang et al. 2017), RAF were less diverse than SAF. Furthermore, the EMF assemblage involved in active symbiosis was far less diverse than the EMF detected by Illumina or other deep sequencing methods (Pena et al. 2017; Schröter et al. 2019). For example, Pena et al. (2017) found approximately 10 to 15 EMF species colonizing root tips per plot, while Schröter et al. (2019), through pyrosequencing, detected approximately 50 EMF species in the same plots. Our EMF results were in a similar range. The high number of OTUs is partly due to methodological bias (Castaño et al. 2018; Nilsson et al. 2019) resulting in species overestimation. Furthermore, the choice of the barcoding sequence, such as ITS or LSU, affects estimates of diversity (Xue et al. 2019). However, it should be noted that consistent response patterns of fungal diversity to environmental factors were detected, irrespective of the method used for fungal community analysis (Xue et al. 2019). Here, the enhanced EMF species richness discovered in the SAF and RAF compared to EMF colonizing root tips likely reflects the ability of EMF, which are not engaged in active symbiosis, to live as saprotrophs in soil or on root surfaces (Iwański and Rudawaska, 2007; Kohler et al. 2015; Lindahl and Tunlid, 2015; Phillips et al. 2014).

According to ecological theory, stress reduces diversity by filtering out species that can tolerate harsh environments (Chase, 2007). This is supported for soil fungi, including ectomycorrhizal communities (Glassman et al. 2017; Schröter et al. 2019; Štursová et al. 2014). For example, along a biogeographic gradient in temperate forests, fungal assemblages were generally less diverse in dry and more acidic soil than in more humid and nutrient-rich soil (Goldmann et al. 2016; Pena et al. 2017; Schröter et al. 2019). Therefore, we anticipated here that P fertilization would result in stress relief and lead to more species-rich, diverse assemblages. While no effect on EMF or SAF richness or diversity was found, P fertilization caused a moderate

decrease in RAF richness in the organic layer, in contrast to our initial hypothesis. Analyses of taxonomic fungal community composition did not reveal any significant response to the fertilization treatments, indicating that the taxonomic dissimilarities and species turnover among the forests dominated small effects at the OTU level.

As outlined in the introduction, high N inputs often cause reductions in fungal diversity and shifts in the community toward nitrophilic assemblages (Bahr et al. 2013; Cox et al. 2010; Lilleskov et al. 2002, 2008; Suz et al. 2014; de Witte et al. 2017). Field studies in temperate forests also identified soil N as an important driver of RAF composition (Lilleskov et al. 2019; Nguyen et al. 2020; Schröter et al. 2019). In other studies, N deposition did not influence fungal community composition (Lilleskov et al. 2019; Purahong et al. 2018). Similarly, we did not observe effects of N addition on the fungal assemblages, irrespective of whether the fungi in soil or those in direct contact with the roots were inspected. Upon N fertilization, less C is allocated belowground to ectomycorrhizal fungi associated with roots (Högberg et al. 2017, 2020). Reduced C availability to the EMF reduces root P uptake (Clausing et al. 2021). These physiological feedback controls might have caused decreased root P concentrations after N addition without requiring strong reshaping of the fungal assemblage. In conclusion, our hypothesis that P fertilization increases fungal richness in P-poor soil and shifts fungal communities to those found in P-rich soil was not supported by our results.

N and P inputs affect the phylogenetic and functional compositions of fungal assemblages

Phylogenetic composition carries information on ecological assembly processes because high relatedness between members of a community suggests similar ecological requirements or functions (Cavender-Bares et al. 2009; Pausas and Verdú, 2010). Information on functions is important to better understand the assembly processes of soil microbes (Nannipieri et al. 2019). Treseder and Lennon (2015) analyzed the fungal traits required for nutrient cycling (e.g., phosphatases and ammonium transporters) in fungal genomes. They found that these potential traits were more conserved, in terms of gene counts, in phylogenetically more closely related taxa (up to the subphylum level) than in the more distant ones (Treseder and Lennon, 2015). Therefore, we reasoned that adaptation of fungal community composition to enhanced N or P inputs might be traceable after aggregating OTUs at a higher classification level, i.e., at the order level. However, in contrast to our hypothesis that N fertilization shifts fungal taxa toward more nitrophilic EMF communities, we observed increases in only Hypocreales (mainly saprotrophic and pathotrophic members) in soil. A novel result of our study was that P fertilization affected mycorrhizal orders (Russulales, Boletales) of both SAF and RAF.

Members of the Russula lineage (*Russula* sp., *Lactarius* sp.) were dominant in our study forests (this study; Clausing et al. 2021; Zavišić et al. 2016). All known members of the Russulales are ectomycorrhizal fungi and are very common in temperate beech forests (Buée et al. 2005; Lang et al. 2011; Taylor et al. 2000). *Russula* and *Lactarius* spp. lack extensive extramatrical hyphae and thus absorb nutrients from their immediate surroundings (Agerer, 2001). Therefore, we assumed that elevated inorganic nutrient availability might favor this fungal genus. However, Russulales showed a significant decrease in response to the combined P + N treatment and increased during spring in soils fertilized only with P. Similar to our study, Mason et al. (2020) found subtle increases in Russulales after P fertilization (5 years) in an LP forest (Ohio, USA). Nicolás et al. (2017) found no significant effects of N fertilization on *Russula* sp. in a boreal forest. However, root colonization and sporocarp formation of *Russula* sp. increased significantly after strong long-term disturbance by high N input (16 years, 170 kg N ha^{-1} a^{-1}) (Avis et al. 2003). Therefore, various *Russula* spp. were classified as nitrophilic species (Avis, 2012). Our results indicate that N availability alone was not decisive. Rather, the N:P ratio regulated the relative abundance of this important fungal order, as Russulales declined significantly when high N addition was accompanied by high P availability.

P fertilization had the most pronounced effects on Boletales, especially in the RAF. Long-distance rhizomorphs and a thick hyphal mantle characterize members of the Boletales (Agerer, 2001). The relative abundance of Boletales increased almost two-fold upon P addition. This result was surprising because investment in high-biomass fungi is considered profitable in nutrient-limited ecosystems in terms of accessing distant resources (Hobbie and Agerer, 2010). For example, Almeida et al. (2019) found increases in *Imleria badia* (formerly known as *Boletus badius*) hyphae accessing apatite (a recalcitrant P source) in N-fertilized soil but not if the N fertilized soil was amended with easily available P sources. Therefore, they argued that *Imleria* is a P-efficient species that responds to an enhanced P demand of the tree (Almeida et al. 2019). However, our results do not support this suggestion because the roots of N-fertilized plots showed decreases in P, which would support an enhanced tree demand under these conditions, whereas the increases in Boletales occurred only in P- or P + N-fertilized plots. Boletales accumulate P in the hyphal mantle and store P in polyphosphate granules in the mycelium (Kottke et al. 1998). One possibility is that the increases in Boletales were responsible for the observed P accumulation in roots in the P-fertilized plots, but it is also feasible that Boletales used P for their own requirements.

In addition to fertilizer treatments, the soil layers also showed differences in the fungal orders present, especially for saprotrophic fungi. Other studies also reported shifts in fungal composition with increasing soil depth (Peršoh et al. 2018; Toju et al. 2016). For example, Toju et al. (2016) found a lower relative abundance of Russulales in the organic layer than in deeper horizons. However, this observation deviates from our results. We found that EMF did not vary between the layers (this study; Clausing and Polle 2020), whereas fungal orders showed significant differences between the organic layer and the mineral soil. In general, the relative abundance of saprotrophic taxa was lower in the RAF than in the SAF. This pattern reflects different nutritional strategies of saprotrophs and mycorrhizal fungi. Saprotrophs prefer environments where bound nutrients can be unlocked from organic compounds, while mycorrhizal fungi mine the mineral layer for inorganic compounds and rely on plant-derived carbohydrates.

An important result of our study was that the relative abundance of saprotrophic fungi was reduced in response to both N and P fertilization. This observation might imply that enhanced availability of mineral nutrients occurs with a trade-off in saprotrophic potential. Future studies should address this proposition by analyzing enzymatic activities. The shift toward symbiotrophs suggests that inorganic nutrient addition might have led to a competitive advantage for the growth of ectomycorrhizal fungi because they obtain carbohydrates from their host, while free-living saprotrophic fungi need to degrade organic compounds to access C. Similarly, field experiments showed that enhanced ectomycorrhizal fungal growth also results in enhanced phosphodiesterase activities and thus higher P_{lab} availability (Müller et al. 2020). Pot experiments under controlled conditions revealed that ectomycorrhizal diversity fostered P uptake efficiency (Köhler et al. 2018), and in forest soils, ectomycorrhizal P uptake efficiency was further related to P_{lab} availability (Clausing and Polle, 2020). These results suggest that P_{lab} availability drives a positive feedback mechanism for plant nutrition. The increase in the relative abundances of symbiotrophic to saprotrophic fungi upon N or P addition might indicate an advantage in capturing mineral nutrients under those conditions. In contrast to enhanced P_{lab} availability, enhanced N_{min} availability resulted in a decrease in root P contents. The shift away from saprotrophic toward symbiotrophic activities may have resulted in lower P mineralization, thereby decreasing P availability and contributing to the reduced root P content in the N-fertilized plot. These considerations underline the important role of saprotrophic fungi in the mineralization of organic P.

Conclusion

Revisiting our hypotheses, we reject our first postulate that P fertilization of P-poor soil leads to increased EMF or RAF richness and diversity and shifts the community composition toward those present in P-rich beech forests. Instead, we found that P fertilization caused a decrease in RAF richness and an increase in the relative abundance of Boletales. At the level of distinct taxa (OTU-based), these shifts were not detectable, indicating that individual responses were diminutive but spread across a group of related species, thus aggregating to measurable effects at higher classification levels. Since Boletales are known for their ability to sequester P (Kottke et al. 1998), our results support the concept of phylogenetic trait conservatism (Powell et al. 2009). Thus, our results may shed light on the apparent ectomycorrhizal community stability in response to nutrient inputs as species-rich fungal assemblages may distribute the response among related members. This suggestion needs to be tested in future studies and may provide an ecological explanation for a frequently observed phenomenon (Lilleskov et al. 2019). We also reject the hypothesis that N fertilization, at least when applied at moderate doses for a relatively short period of time (2.5 years), affects the fungal communities, thus supporting the notion that EMF in temperate beech forests are relatively resistant to N inputs (Lilleskov et al. 2019). Since the relative abundance of SAF and RAF symbiotrophic fungi increased under N fertilization while root P contents declined irrespective of the soil P content, N inputs may lock P in fungal biomass, with negative consequences for tree P nutrition. We speculate that Russulales play a prominent role in this regard because the negative feedback of N fertilization on root P was compensated for by additional P application and was accompanied by decreases in Russulales. Overall, our results emphasize the importance of distinguishing different habitats and including the major nutrients N and P to better understand the drivers of fungal communities in relation to nutrient cycling.

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Author contributions SC and AP conceived the study. FL and JK realized the fertilization experiment, maintained the plots and contributed field data. SC conducted field and laboratory measurements. LEL conducted EMF sampling and analyses. HYF determined CN. SC, LEL, DJ, DS, RD, and AP analyzed data. SC compiled the data and wrote the first manuscript draft. AP revised the draft. All authors contributed to, commented, and agreed on the final version.

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Data availability The datasets generated for this study can be found in the Dryad digital repository (https://doi.org/10.5061/dryad.rv15dv473). The sequences for identified mycorrhizal fungi are available in NCBI GenBank under accession numbers MT859114 to MT859131. The Illumina MiSeq sequences of fungal ITS2 can be found in the Sequence Read Archive (SRA) of NCBI under Bioproject PRJNA680926.

Declarations

Conflict of interest The authors declare no competing interests.

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