Strong family- and guild-specific responses of arbuscular mycorrhizal fungi to long-term deficiencies and imbalances of N, P and K

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Summary

- Many agroecosystems face nitrogen (N), phosphorus (P) or potassium (K) deficiencies due to imbalanced or insufficient nutrient replenishment after plant biomass harvest. How this affects the symbiosis between plants and arbuscular mycorrhizal fungi (AMF), and the abundance of exploration-based AMF guilds (i.e., rhizophilic, edaphophilic, ancestral) remains largely unknown.
- We studied a 70-year nutrient-deficiency experiment in a managed grassland in central Austria, where aboveground biomass was harvested three times annually. Nitrogen, phosphorus and potassium were fully, partially, or not replenished, causing strong nutrient deficiencies and imbalances. We analysed AMF communities in soil and roots by DNA/RNA amplicon sequencing and fatty-acid biomarkers, alongside soil and plant community changes.
- Soil AMF communities were primarily affected by N and P deficiencies, while root AMF communities were most susceptible to K deficiency, with a 50% reduction in root AMF biomass. We observed distinct, guild- and family-specific responses: edaphophilic AMF declined with nitrogen deficiency, while rhizophilic fungi decreased with phosphorus and potassium deficiencies. Families within each guild, particularly ancestral ones, exhibited differential responses, indicating complementary nutrient specializations at the family level.
- Our findings underscore the previously unrecognized role of K deficiency in AMF symbiosis, and suggest the existence of nutrient-related functional subgroups within exploration-based AMF guilds.

Keywords: ancestral, AMF abundance and community composition, arbuscular mycorrhizal fungi, edaphophilic, exploration-based functional guild, long-term nutrient deficiencies and imbalances, managed grassland, rhizophilic

Introduction

Arbuscular mycorrhizal fungi (AMF) are one of the oldest and most important plant root symbionts (Sochorová *et al.*, 2016; Thirkell *et al.*, 2016; Brundrett & Tedersoo, 2018; Hawkins *et al.*, 2023), which form mutualistic associations with almost all plant species in croplands and managed grasslands. They supply essential soil nutrients such as N, P and K to their host plants (Smith & Smith, 2011; Berruti *et al.*, 2016; Bravo *et al.*, 2017; Igiehon & Babalola, 2017), along with other benefits such as protection from pathogens (Borowicz, 2001), improving hydraulic conductance at the soil-root interface (Abdalla *et al.*, 2023), and improving the physical soil structure by facilitating soil aggregation (Rillig, 2004; Rillig & Mummey, 2006). It is estimated that AMF-associated plants transfer about 6% of their net primary production on to their fungal partners, accounting for a total global C flux of about one Gt C year^{-1} into the soil (Frey, 2019; Hawkins *et al.*, 2023) substantially contributing to the global buildup of soil C stocks (Rillig, 2004; Godbold *et al.*, 2006; Hawkins *et al.*, 2023; Kuyper & Jansa, 2023).

AMF are known to be sensitive to changes in soil nutrient availability by fertilization (Johnson *et al.*, 2015). However, it is currently unknown how AMF respond to the nutrient deficiencies and imbalances, commonly observed in many agricultural soils worldwide (Manning, 2012). Estimates indicate that, as of the year 2000, global soils faced nutrient deficiencies amounting to 18.7 N, 5.1 P and 38.8 K (kg ha^{-1} yr^{-1}) on 59%, 85% and 90% of all harvested area, respectively (Tan *et al.*, 2005). These deficiencies and subsequent elemental imbalances are partly caused by insufficient fertilization which does not fully replenish nutrients removed from soil by harvesting plant biomass (Manning, 2012). Additionally, atmospheric N deposition rates have been increasing due to anthropogenic activities, which is not matched by similarly increasing inputs of P, widening the N:P imbalance across all ecosystems (Peñuelas *et al.*, 2013; Lu & Tian, 2017) . This situation is aggravated by increasing K deficiencies in many managed soils worldwide, due to a disproportional lack of K replenishment compared to N and P, as evident in particular in many managed soils from Africa or China (Tan *et al.*, 2012; Manning, 2012; Zörb *et al.*, 2014).

Under low nutrient availabilities, AMF are indispensable partners for plants because their extraradical hyphal networks are far more efficient in accessing inorganic nutrients (in particular P) compared to plant roots (Smith & Smith, 2011). It is assumed that plants reduce their support for AMF when these nutrients are available sufficiently or in excess so that they can be taken up efficiently by the root system (Hoeksema *et al.*, 2010). For example, high

levels of P fertilization have frequently been found to result in decreasing root colonization by AMF (Beauregard *et al.*, 2010; Cavagnaro, 2015; Propster & Johnson, 2015; Liu *et al.*, 2016). However, it is not only absolute quantities but also relative proportions (or stoichiometry) of available nutrients that are decisive (Johnson, 2010; Smith & Smith, 2011; Johnson *et al.*, 2015) : While AMF tend to respond positively to N fertilization under low P availability, they respond negatively when soil P availability is above a certain threshold (Johnson *et al.*, 2015; Jiang *et al.*, 2018; Han *et al.*, 2020). In contrast to inorganic fertilizers, applying organic fertilizers has often been found to improve AMF growth (Cavagnaro, 2015; Yang *et al.*, 2018). While the mechanisms behind these observations are not yet fully understood, the slow release of nutrients from organic fertilizers may be beneficial for AMF (Yang *et al.*, 2018). Strikingly, and in stark contrast to its importance, almost nothing is known about the response of AMF to absolute or relative changes in K availability.

Under long-term nutrient deficiencies, indirect effects on AMF through ecosystem feedbacks become additionally important. For example, changes in plant community composition or soil physicochemical properties (e.g., pH, water or organic matter content (Jamiołkowska *et al.*, 2018), are induced by nutrient deficiencies. Particularly, pH is believed to play an eminent role for AMF distribution and abundance (Clark, 1997; Van Aarle *et al.*, 2002; Van Geel *et al.*, 2018; Davison *et al.*, 2021). Such indirect effects are naturally intermingling with direct effects of nutrient deficiencies on AM fungi, and difficult to disentangle.

At the same time, AMF communities may exert strong feedbacks on ecosystem properties, for example by shaping plant communities through changes in plant competitiveness, influencing soil physical structure, and building up soil C stocks via their mycelia. The proportion and extent of fungal mycelia reaching into the soil - a trait of potential ecosystem relevance - is considered to be phylogenetically conserved at the family level (Hart & Reader, 2002; Chagnon *et al.*, 2013). AMF families have thus recently been classified into ‘guilds’ based on exploration traits (Phillips *et al.*, 2019; Weber *et al.*, 2019): The ‘edaphophilic guild’ includes families building large extraradical (i.e. outside plant roots in the soil) but only little intraradical (i.e. inside plant roots) mycelia (e.g. Gigasporaceae). The ‘rhizophilic guild’, in contrast, encompasses families with large intraradical but little extraradical mycelium (e.g. Glomeraceae), and the ‘ancestral guild’ (e.g. Acaulosporaceae) comprises families that have both low intraradical and extraradical biomass (Maherali & Klironomos, 2007; Weber *et al.*, 2019). Different functional traits are ascribed to each of these guilds based on the structure of their mycelia and on taxon- and family-specific observations (Hart & Reader, 2002; Maherali & Klironomos, 2007; Treseder *et al.*, 2018; Weber *et al.*, 2019): edaphophilic taxa are thought

to be more efficient in supporting plant nutrient uptake and exhibit a higher soil C sink strength due to their larger share of soil-based mycelium (Chagnon *et al.*, 2013), while rhizophilic taxa are more effective in pathogen defense and conferring stress-tolerance (Chagnon *et al.*, 2013; Weber *et al.*, 2019). Therefore, shifts in the proportion of these AMF guilds in response to nutrient deficiencies and imbalances may significantly impact vital ecosystem functions, such as plant nutrient acquisition and soil C sequestration.

Although most plants in agroecosystems are associated with AMF it is currently unknown how AMF communities will respond to the increasing nutrient deficiencies and imbalances. Only few studies investigated the long-term effect of nutrient deficiencies on AMF abundance and community composition in managed grasslands or agricultural fields (Qin *et al.*, 2015; Heyburn *et al.*, 2017; Zheng *et al.*, 2018). Furthermore, there is almost nothing known on the behavior of AMF exploration-based functional guilds under nutrient deficiency and imbalances. Additionally, while most studies focused on the effect of P as well as N, almost nothing is known about how AMF respond to K deficiency and imbalances between K and other nutrients.

In this study, we aimed to investigate the impact of over 70-year of nutrient deficiencies and imbalances of N, P and K on arbuscular mycorrhizal fungal communities and exploration-based functional guilds in a managed grassland. Specifically, we asked the following questions:

1. How do long-term nutrient deficiencies and imbalances of N, P and K influence abundance and community composition of AMF?
2. Do single or combined deficiencies of nutrients cause shifts in the proportion of AMF exploration trait-based functional guilds?
3. Are responses of AMF communities to long-term nutrient deficiencies and imbalances linked to responses of plant communities and soil properties?

We addressed these questions in a long-term nutrient deficiency experiment in a grassland site in Austria, which was launched in 1946 (representing more than 70 years of nutrient manipulations). We analyzed the effects of single and multiple nutrient deficiencies of N, P and K on AMF abundance and community composition. For investigating the effect of pH we also included plots that were regularly limed. We investigated the intra- and extraradical part of the mycelium by analyzing both soil and root samples for AMF, as well as assessing the effect on exploration-based AMF functional guilds. Furthermore, we examined the plant

community composition and various soil abiotic factors to identify potential links between these parameters and changes in AMF communities.

Materials and Methods

Study site

The study was conducted at a long-term nutrient deficiency experimental grassland site of the Agricultural Research and Education Centre Raumberg-Gumpenstein in Admont, Styria, Austria (47° 34’ 58’’ N, 14° 27’ 02’’ E; 635 m a.s.l.). This experimental grassland was established in 1946 on a hay meadow, with a continuous three-cut management regime since the start of the field experiment. According to the WRB-system, the soil at the experimental site is classified as Gleyic Fluvic Dystric Cambisol with no supplementary qualifiers (FAO, 2015). The long-term average annual precipitation is 1227 mm, and long-term mean annual air temperature is 6.8 °C.

Nutrient deficiency experiment

In total, 24 nutrient-fertilization treatments (4 replicates each, in size of 2.9 m × 7.1 m) are established in a randomized block design. The fertilization treatments include different variations of inorganic (P, N, and K) or organic (solid manure and liquid slurry). Furthermore, some plots receive a liming treatment in addition to inorganic fertilizers. Nitrogen is applied as calcium ammonium nitrate (NH_4NO_3 + $CaCO_3$) in the amount of 80 kg N $ha^{-1}year^{-1}$ (half of it applied in spring, and the half after the first cut). Phosphorus was applied as Thomas phosphate until 1997, as hyperphosphate at an equivalent rate of 34.9 kg P ha^{-1} $year^{-1}$, and potassium fertilization as potassium chloride at an equivalent rate of 99.6 kg K ha^{-1} $year^{-1}$. These two nutrients are each fertilized in autumn. Some variants are additionally fertilized with mixed lime ($CaCO_3$ + $MgCO_3$) applied in the equivalent amount of 928 kg Ca ha^{-1} every third year in autumn. Solid manure (37% N, 12% P, 51% K) and liquid slurry (35% N, 1% P, 64% K) are added in a quantity of 15 t ha^{-1} (in autumn) and 40 t ha^{-1} (half applied in spring and half after the first cut), respectively. While in the fully fertilized treatment, all three nutrients are provided, other treatments are not fertilized by one or more elements in the nutrient-deficient treatments. Consequently, while aboveground plant biomass and nutrients have been continuously removed, only part of the nutrients has been replenished by fertilization. In addition, an unfertilized control treatment never received any fertilizer while still being mowed three times a year since 1946. This practice has led to different types of long-term nutrient deficiencies in this grassland. Fig. **S1** shows 14 treatments that have been selected for this experiment and their distribution on the site.

Sample collection

We collected the soil samples to 9.5 cm depth in July 2019, using 2 cm diameter corers, and pooled 8 to 11 randomly distributed cores per plot for a composite sample of around 300 g. The composite sample was then carefully sieved to 2 mm, and roots were separated from the soil. Fine roots were washed and cleaned. Subsamples for DNA and RNA extraction from both mixed root and soil samples were frozen in liquid-N2 and kept at -80 °C. After lyophilization, root samples were ground in a cryomill with liquid N_2 and both soil and root samples were kept at -20 °C for further analysis.

Soil characterization

Soil pH (H_2O) was measured in a 1:5 (w:v) soil slurry. Soil water content was evaluated gravimetrically, after drying soil samples at 105 °C for 24 h. Soil aliquots were dried at 60 °C for 72 h, weighed and finely ground for subsequent analysis of bulk total C and N contents by EA-IRMS (EA 1110; CE Instruments, Milan, Italy), coupled to a Finnigan MAT Delta Plus IRMS (ThermoFisher Scientific, Waltham, MA, USA).

Inorganic phosphate was assessed photometrically by the phosphomolybdate blue reaction (Schinner *et al.*, 1996), in 0.5M $NaHCO_3$ extracts, with or without prior alkaline persulfate oxidation to estimate total dissolved P and dissolved inorganic P, respectively (Doyle *et al.*, 2004). Additionally, ammonium was quantified by in 1 M KCl extracts spectrophotometrically by the Berthelot reaction (Tecan Infinite M200fluorimeter, Grödig, Austria) (Kandeler & Gerber, 1988; Hood-Nowotny *et al.*, 2010a). Nitrate was also measured in 1 M KCl soil extracts by the VCl_3 reduction and Griess reaction (Miranda *et al.*, 2001; Hood-Nowotny *et al.*, 2010b). Soil total free amino acids were determined by the fluorometric method based on Jones et al. (2002) (Jones *et al.*, 2002). Total dissolved N and dissolved organic C were assessed in 1 M KCl soil extracts by TOC/TN-analyzer (TOC-V CPH/TMN-1, Shimadzu, Japan), and dissolved organic N was calculated by subtracting the sum of ammonium and nitrate from total dissolved N.

Microbial C, N and P were measured following the chloroform fumigation-extraction technique (Vance *et al.*, 1987), with modifications. Briefly, 4 g of soil from each replicate was weighed in two containers. To the first set of containers, 30 mL of 1 M KCl solution (for C and N) or 0.5 M $NaHCO_3$ (for P) was added and shaken for 1 h before filtering through ash-free cellulose filters and extracts were kept frozen at −20 °C for later analyses.

The other set was fumigated with chloroform for 48 h in the dark and then extracted similarily. Dissolved organic C, total dissolved N and P were measured in the extracts as described above. Microbial biomass C was calculated by dividing the difference between fumigated and non-fumigated samples by 0.45, and microbial biomass N by 0.54; no factor was used for microbial biomass (McDonald *et al.*, 1998).

Plant community composition

The plant community composition was determined visually in 2015 according to the modified method of Braun-Blanquet (1951) (Braun-Blanquet, 1951) described in Peratoner and Pötsch (2019) (Peratoner & Pötsch, 2019). The plant species were identified taxonomically based on Fischer et al. (2008) (Fischer *et al.*, 2008). The experimental plots were harvested in 2018 using a motorized bar mower and the green mass yield was determined directly in the field. A representative sample was then taken from each plot to determine the dry matter content and to carry out forage analyses using standardized methods by ALVA (1983) and VDLUFA (1976) (VDLUFA, 1976; ALVA, 1983).

Neutral fatty acid biomarkers as proxy for AMF biomass

Neutral fatty acids (NLFAs) in soil and root samples were evaluated by the procedure described in Gorka *et al.* (2023) (Gorka *et al.*, 2023). We extracted total lipids from soil and root samples with a mixture of chloroform, methanol, and 0.15 M citrate buffer (pH = 4; v:v:v = 1:2:0.8) (Bligh & Dyer, 1959). Neutral lipids were then separated from other lipid classes by elution from 96-well silica plates (Strata SI-1 Silica, 55 µm, 70 Å, 50 mg/well, Phenomenex, Canada) (Buyer & Sasser, 2012; Drijber & Jeske, 2019) with chloroform with 2% ethanol. Subsequently, NLFAs were released from the lipids and transmethylated by alkaline methanolysis. After fractionation, Methyl-nonadecanoate (10 µg sample^{-1}) was added to samples as an internal standard for quantification of fatty acids by GC (7890B Agilent Technologies, USA) coupled to a TOF-MS (Pegasus BT, LECO, USA). NLFA 16:1ω5 was identified by comparison of chromatographic retention times to external fatty acid methyl ester standards (37 Component FAME mix, and BAME CP mix, Supelco, Austria), and by comparison to the NIST library of mass spectra. We used NLFA 16:1ω5 as a proxy for soil and root AMF biomass (Kaiser *et al.*, 2015).

AMF amplicon sequencing

AMF community composition was assessed by RNA (cDNA) amplicon sequencing (after DNAse treatment of the total nucleic acid extracts and reverse transcription to cDNA, using

SuperScript™ III Reverse Transcriptase from Invitrogen) and DNA amplicon sequencing of 18S rRNA gene by the procedure described in Bukovská et al. (2021) (Bukovská *et al.*, 2021), using WANDA (CAGCCGCGGTAATTCCAGCT) and AML2 (GAACCCAAACACTTTGGTTTCC) primers.

We conducted both RNA and DNA amplicon sequencing (18S rRNA gene) in soil samples, whereas only DNA amplicon sequencing (18S rRNA gene) was performed in root samples. In soil samples, total nucleic acids were extracted by phenol-chloroform procedure (Angel *et al.*, 2012), whereas Qiagen DNeasy Plant Mini Kit was used to extract DNA from root samples. The amplicons were dually indexed with Nextera XT indexesand sequenced on Illumina MiSeq 2 × 300 platform (Nilsson *et al.*, 2019; Bukovská *et al.*, 2021). We used Seed software and external software packages to process sequencing results (Větrovský & Baldrian, 2013; Jansa *et al.*, 2020). In detail, a minimum of 20 bp overlap was considered to pair raw sequence reads and the sequences that had average quality scores less than 30 were removed (Bukovská *et al.*, 2021). After that, primers were eliminated from sequences, and chimeras filtered out. We then clustered the sequences at a 97% similarity threshold into operational taxonomic units (OTUs) and these were identified by the SILVA database, retaining only OTUs belonging to the Glomeromycota (Bukovská *et al.*, 2021). Relative abundances of OTUs per sample were calculated at the genus level (Bukovská *et al.*, 2021). AMF families were categorized based on their known growth traits (hyphae allocation in soil and roots) into three main guilds: rhizophilic, edaphophilic, and ancestral (Parniske, 2008; Powell *et al.*, 2009; Weber *et al.*, 2019).

DNA and RNA amplicon sequencing of the 18S rRNA gene showed comparable pattern of soil AMF communities (Fig. **S2**). However, DNA amplicon sequencing did not yield sufficient sequencing depths regarding AMF communities in soil samples, because the WANDA primer also amplified lots of tardigrades, nematodes, and other invertebrates in soil. As the sequencing depth yield was far better for RNA amplicon sequencing, we decided to use RNA amplicon sequencing of the 18S rRNA gene to assess soil AMF community composition. The corresponding analysis of DNA sequencing, is provided in the supplementary material (Figs **S9**, **S10**).

Statistical analysis

All statistical analyses were carried out in R (version 4.3.3), plots were generated using the package “ggplot2” (version 3.4.4) (Hadley, 2016). Following checks for normality and

homogeneity of variance, we performed Analysis of variance (ANOVA) for data meeting these assumptions, Tukey's *post-hoc* test for treatment comparisons. For non-normally distributed data, we used Kruskal–Wallis H test and Bonferroni-corrected paired samples t-test as *post-hoc* test. Multivariate analysis was conducted using "Vegan" package (version 2.6-2) in R (Oksanen *et al.*, 2007). We analyzed the influence of long-term nutrient deficiencies on soil and root AMF and plant communities by correspondence analysis (CA). CA1 scores, derived from this analysis, served as indicators of soil and root AMF and plant community compositions.

We tested the relationship between AMF abundance and community composition, with various environmental factors by Pearson's and Spearman's correlation for normally and non-normally distributed variables, respectively.

We used multiple regression to evaluate the influence of environmental factors on soil and root AMF biomass and community composition. Hierarchical partitioning of R^2 was used determine the variance explained independently or jointly by those environmental variables (see Notes **S1**).

Results

AMF biomass was driven by N and P in soils, but by K availability in roots

AMF biomass (using 16:1ω5 NLFA as a proxy) in soil and roots was strongly affected by long-term nutrient deficiencies and fertilization regimes. N, P and K deficiencies had strong and distinct effects on AMF biomass (Fig. **1a,b**). The response of AMF biomass in roots and soil (intra- and extraradical mycelia, respectively) was driven by different element deficiencies. While N deficiency significantly increased AMF biomass in soil (F=27.51, P<0.001; Fig. **1a;** Table **1**), its effect on AMF biomass in roots was less pronounced (F=4.13, P=0.053). P deficiency had a weak negative influence on AMF biomass in soil (F=3.04, p=0.09), but no effect in roots. However, P deficiency had a significant positive effect on soil AMF biomass per unit of plant biomass, in particular when soils were K fertilized (F=27.51, P=0.005; Fig. **1d**; Table **1**). We also observed a significant interaction between the effects of N and K on soil AMF biomass (F=9.37, P<0.01; Table **1**). The lowest AMF biomass was observed in soils that received N fertilization but were at the same time K and P deficient (Fig. **1a**). In contrast to soil samples, K availability was the strongest predictor for AMF biomass in roots. K deficiency significantly diminished AMF biomass in roots (F=13.35, P<0.01; Fig. **1b**; Table **2**).

AMF community composition differed systematically between soils and roots

In both soil and root samples, we found 14 AMF genera, belonging to eight families (Fig. **2**). Roots were strongly dominated (approximately 75 – 99%) by AMF families and genera belonging to the rhizophilic guild (mostly *Dominika*, *Rhizophagus*, and *Glomus*; Fig. **2b**). In soil samples, AMF families ascribed to the ancestral or edaphophilic guilds made up a considerable proportion of the total abundance (approximately 20 – 75%), especially in inorganically fertilized plots (Fig. **2a**).

A multivariate correspondence analysis (CA) revealed that root samples were clustered together and significantly separated from soil samples over all treatments (PERMANOVA: F=81.35, P<0.001; Fig. **3a**). This analysis shows that genera such as *Rhizophagus*, *Dominikia* and *Glomus*, belonging to the family Glomeraceae, were relatively more abundant in root samples, whereas other genera such as *Scutellospora* and *Acaulospora*, belonging to ancestral and edaphophilic families were more abundant in soil samples. This clustering (separation of root and soil samples) was also visible when comparing soil and root DNA (Fig. **S3**), which rules out that it could have been caused by differences between DNA and RNA abundances in roots and soil, respectively.

Long-term fertilization regimes affected AMF community compositions in both soil and roots

While the axis with the largest explanatory power separates root from soil samples (CA1, explains 36.58% of the variability of the data), the second most explanatory axis (CA2, explains 21.89% of the variability of the data) is driven by the fertilization treatments (Fig. **3**). There is a clear separation between lime and organically fertilized plots on the one hand, and the inorganically fertilized plots on the other hand, which is consistent across root and soil AMF communities (Figs **3b, S3b**).

Similar to AMF biomass, AMF community composition was linked to N and P availability in soils, but to K availability in roots

To better understand the effect of N, P, and K deficiencies in each sample type, we performed separate multivariate analyses focusing only on inorganic treatments in the soil or root communities (Fig. **4**). This analysis revealed that nutrient deficiencies affected AMF community composition differently in roots and soils. In soil samples, P-deficient treatments clustered together, except for the completely unfertilized treatment (Fig. **4a**). Furthermore, treatments with N deficiency were separated from P-deficient treatments. The treatments

fertilized with N and P (NP and NPK, respectively) were separated from the other treatments. PERMANOVA analysis indicated a statistically significant difference between P deficient, N deficient, and P plus N fertilized clusters (PERMANOVA: F=6.53, P<0.001).

In contrast, AMF community composition in root samples was mainly linked to K availability (Fig. **4b**). Roots in K-deficient plots hosted completely different communities compared to K-fertilized plots. Based on PERMANOVA analysis, it showed a statistically significant difference between K deficient and fertilized clusters (PERMANOVA: F = 5.23, P<0.01) (Fig. **4b**). This pattern of a dominant effect of N and P availabilities on soil, but of K availability on root AMF communities are in line with our findings on AMF abundance (Fig. **1a,b**).

Strong response of AMF guilds and families to inorganic nutrient deficiencies

We observed strong and clear responses of AMF families and guilds to nutrient deficiencies, that were mostly consistent across root and soil samples. For example, plots that never received P fertilization, and were thus P deficient, showed lower relative abundances of Paraglomeraceae (in both roots and soil) and Claroideoglomeraceae (in roots), two families belonging to the rhizophilic guild (highly significant at P<0.001; Tables **1**, **2**; Fig. **S5**). In soil samples, rhizophilic taxa responded significantly negatively to P deficiency at the guild level. At the same time, P deficiency increased the relative abundance of the ancestral guild in soil samples, mostly driven by the decrease of Ambisporaceae (P<0.001; Tables **1**, **2**; Fig. **S6**).

In contrast, N deficiency did not affect members of the rhizophilic guild in neither soil nor root samples, but decreased Gigasporaceae, a family within the edaphophilic guild, in both sample types (Tables **1**, **2**; Fig. **S7**) with the effect being more significant in root samples. In addition, N deficiency led to a shift between families within the ancestral guild, by significantly increasing Acaulosporaceae and reducing Ambisporaceae in soil samples (Table **1**; Fig. **S6**). A similar trend was visible in root samples (Table **2**; Fig. **S6**).

While P deficiency reduced less dominant rhizophilic families such as Paraglomeraceae and Claroideoglomeraceae, K deficiency had a strong negative effect on the dominant rhizophilic family, Glomeraceae, in both roots and soils. Glomeraceae AMF were generally lower in K-deficient plots compared to K-fertilized ones. This shaped a significantly negative response of the rhizophilic guild in total to K deficiency (Tables **1**, **2**; Fig. **S5**). At the same time, K deficiency led to an increase in ancestral guilds, mainly Archeasporaceae and Ambisporaceae in both roots and soils and edaphophilic guild in roots (Tables **1**, **2**; Figs **S6**, **S7**).

Changes in soil AMF communities were linked to changes in plant communities

Besides AMF community composition, long-term nutrient deficiencies also influenced plant communities. A correspondence analysis of aboveground vegetation data showed that plant community composition was, similar to soil AMF communities, mostly influenced by N and P deficiencies, while K deficiency was of minor importance (Fig. **S11**).

To investigate whether shifts in AMF community composition were linked to changes in plant community composition we correlated the sample scores of the CA1 axes, i.e. the axis with the most explanatory power of the correspondence analyses of plant and AMF communities with each other (Fig. **5**). Scores on these axes represent a composite measure for community composition, i.e. two communities with more similar scores are more similar to each other, whereas communities with less similar scores are more dissimilar to each other. If the change of AMF community composition is linked to plant community changes we expect that their CA1 scores are correlated with each other, i.e. that more similar plant communities will host more similar AMF communities.

Our analysis revealed that – when only considering inorganically fertilized treatments - the plant community composition was significantly linked to the soil AMF community composition (R^2=0.48, P<0.001; Fig. **5a**), but not to the root AMF community composition (R^2=0.05, P>0.05; Fig. **5b**). Moreover, pH was significantly correlated with both soil AMF and plant community compositions CA scores (Fig. **5c,d**)

By assessing the correlations across all treatments (i.e., including also organic fertilizers and lime treatments), we observed an even stronger correlation between soil AMF community and plant community (R^2=0.55, P<0.001), and a weak, but still significant, correlation between root AMF and plant communities (R^2=0.27, P<0.001; Fig. **S13**).

AMF communities were positively affected by liming and organic fertilization

Considering all treatments (inorganic, lime and organic treatments), pH exhibited the strongest link to AMF abundance and community composition in soil (Figs **6b**, **S13**). The influence of pH on soil AMF biomass was also demonstrated by their response to the lime treatments. Liming substantially increased soil pH values and had a strongly positive impact on soil AMF abundance (F=86.33, P<0.001), especially in unfertilized plots where it was almost twice as high as in unfertilized plots that received no lime (Fig. **6a**). The positive effect of liming on soil AMF was reduced when PK or NPK fertilizers were added to limed plots (P<0.01 for both PK+Lime and NPK+Lime compared to PK and NPK treatments,

respectively). The effect of liming on root AMF was less pronounced, but still significant (F=6.90, P<0. 05; Fig. **6c**).

Similar to liming, organic fertilization also led to higher AMF biomass in soils (but not in roots) compared to the fully fertilized (NPK) inorganic treatment (Fig. **S18**). The positive effect on soil AMF biomass was most pronounced when solid manure was added (F=6.11, P<0.05) but its combination with liquid slurry lessened this effect (F=8.50, P<0.05; Fig. **S18a**).

AMF biomass is mostly explained by dissolved N concentrations while AMF community composition is linked to plant parameters, pH and organic P.

AMF biomass and community composition were correlated to several soil parameters across the inorganically fertilized treatments and its unfertilized control (Fig. **7**). While soil AMF biomass was most strongly associated to measures of dissolved N (e.g. total dissolved N, ammonium, nitrate), AMF community composition was strongly associated to pH and plant community composition, followed by dissolved organic P (positively), total free amino acids and dissolved organic C (both negatively). In general, there were stronger links between soil parameters and soil AMF than between soil parameters and root AMF (Figs **7**, **S13**).

The hierarchical partitioning of the effects of all measured soil and plant parameters on soil and root AMF abundance showed that the concentration of soil nitrate had the strongest effect across unfertilized and inorganically fertilized plots (Fig. **8a,b**). Upon extending the analysis to include all treatments (inorganic, limed and organic), pH was the parameter with the strongest link to soil AMF abundance. However, nitrate remained the predominant factor influencing root AMF abundance (Fig. **S14a,b**). Aboveground plant biomass and community composition did not play a major role in AMF abundance in both analyses (Figs **8a,b**, **S14a,b**). Soil AMF community composition was, by contrast, most affected by pH and plant community composition (Figs **8c**, **S14c**), whereas root AMF community composition was primarily driven by nitrate, seconded by – if all treatments were considered –plant community composition and pH (Figs **8d**, **S14d**).

Discussion

Prolonged soil nutrient deficiencies disrupt the balance among plants, their AMF partners, and soil nutrient states. Predicting the outcomes of such disruptions is challenging due to the complexity of these interactions. Our findings indicate that 70 years of nutrient removal through biomass harvest significantly impacted AMF communities in managed grasslands,

with distinct fungal families responding differently to specific nutrient deficiencies. These shifts were linked to changes in plant community composition and soil properties, suggesting that plant-fungal interactions can drive system-wide changes.

Soil AMF communities and fungi-to-plant biomass ratios were influenced by N deficiency, but shaped by interactions with P and K deficiencies

AMF are considered crucial for P nutrition of grassland plants, while their role in N nutrition remained obscure (Smith & Smith, 2011). We expected P availability to have a greater impact on AMF communities than N. Contrary to this expectation, we found that N played the most important role for soil AMF biomass. Significantly more AMF were present in N-deficient compared to N-fertilized soils, despite N deficiency reducing aboveground plant biomass. This led to an increased AMF biomass-to-plant biomass ratio, suggesting enhanced carbon transfer to AMF, potentially faciliating fungal uptake and delivery of the limiting nutrient.

Contrary to N, P deficiency had only a minor effect on soil AMF biomass (P=0.09). Similar to N deficiency, P deficiency also significantly increased the ratio of soil AMF-to-plant biomass, though only when K was available (Fig. **1d**). The increase in the soil fungi-to-plant biomass ratio in both N- or P-deficient plots that had received K fertilization was driven by a reduced plant biomass under N or P deficiency, while soil AMF biomass remained largely unchanged (compare red bars in Fig. **1a,c**). However, in K-deficient plots, plant biomass was reduced compared to fully fertilized plots, but remained unaffected by P or N deficiency (blue bars in Fig. 1c), while K deficiency enhanced the negative effects of N fertilization and P deficiency on soil AMF biomass (blue bars in Fig. 1a). Together, this suggests (i) that plant P or N limitation in K-fertilized plots may have led to higher plant support of AMF per unit of (aboveground) plant biomass, as has been suggested for P limitation in general (Beauregard *et al.*, 2010; Propster & Johnson, 2015; Hu *et al.*, 2019), and (ii) this support may have been disrupted under plant K limitation.

Stoichiometry-based concepts, like the 'trade balance' and the 'functional equilibrium' models suggest that AMF symbiosis functionality depends on the N-to-P ratio (Johnson, 2010). If P is not added alongside N, increased N availability leads to a relative P deficiency in plants. Plants would therefore allocate more C to their fungal partners to improve P uptake. However, if P is added, plants will reduce carbon allocation rates towards mycorrhizae in favor of aboveground structures. It has thus been suggested that AMF respond positively to N fertilization in soils when P is low, but negatively when P is abundant (Johnson, 2010; Wipf

et al., 2019). However, our results contradict this pattern, revealing strong interplays among N, P, and K instead. The lowest soil AMF biomass (roughly minus 30% compared to the fully fertilized treatment and minus 50% compared to the unfertilized treatment) was observed when soils had received N but simultaneously lacked both P and K. Conversely, N and P deficiencies together resulted in the highest AMF biomass,(Fig. **1a**). This shows that K plays an important and possibly so far-overlooked role in shaping AMF responses to varying nutrient availabilities.

K deficiency strongly shaped root AMF communities

In contrast to soil AMF, which were mostly affected by N deficiency, root AMF were mostly affected by K availability. AMF biomass in roots was significantly reduced by up to 50% in K-deficient soils. (Fig. **1b**; Table **2**). K is essential for higher plants, impacting carbon and water transport (by facilitating plant transpiration via stomata regulation), photosynthesis, nutrient uptake and stress response (Egilla *et al.*, 2005; Sardans & Peñuelas, 2015). Particularly, K facilitates saccharide transfer from leaf parenchymal cells to the phloem (Deeken *et al.*, 2002; Sardans & Peñuelas, 2021) and is key for the supply of sink organs with photosynthates (Zörb *et al.*, 2014). Therefore, K deficiency likely reduced plant water uptake efficiency, photosynthesis, and photosynthate transport to below-ground plant organs and symbionts. Impaired water transport in plants is indicated by the increased soil water content in K-deficient plots (Fig. **S15b**; Table **S1**). As water content was however not related to AMF biomass in soils or roots (Fig. **7**), we assume that enhanced water contents did not directly affect AMF biomass. Potentially impaired transport of photoassimilates to roots induced by K-deficiency could explain the lower root colonization and the lack of a positive response of AMF in K-deficient plots to P deficiency. The effect of K deficiency was strongest on AMF from the Glomeraceae family, which predominated in roots but were less abundant in soils, explaining the greater impact of K deficiency on root AMF compared to soil AMF.

Few studies have investigated the effect of soil K availability on AMF root colonization. It has been shown that AMF abundance in the roots of an endemic alpine herb was linked to soil K and calcium availability (Casazza *et al.*, 2017). Liu *et al.* (2019) found that mycorrhizal tomato plants had higher AMF colonization under K fertilization. This was driven by higher rates of C allocation towards their roots to promote K uptake by AMF (Liu *et al.*, 2019).K deficiency also boosts reactive oxygen species (ROS) in roots (Cakmak, 2005; Garcia *et al.*, 2017), which could damage AMF in direct proximity. These findings support the positive effect of K on root AMF biomass observed in our study.

Distinct effects of long-term nutrient efficiencies on soil- versus- root-associated AMF community composition

Rhizophilic AMF (particularly from Glomeraceae*)* were more abundant in root samples, while edaphophilic and ancestral AMF predominated in soil (Fig. **2**). This reflects their exploration traits: while rhizophilic AMF produce a relatively higher proportion of hyphae inside the roots, edaphophilic AMF produce relatively more extraradical hyphae (Powell *et al.*, 2009; Weber *et al.*, 2019).

Ancestral AMF are thought to produce low amounts of both extraradical and intraradical hyphae (Powell *et al.*, 2009; Weber *et al.*, 2019). Still, we find them in a quite high proportion in both roots and soil, even outnumbering edaphophilic taxa in soils (Fig. **2**). This, together with our finding about their specific response to nutrient limitations, confirms previous suggestions that ancestral AMF may possess unique functional traits and may play more important ecosystem roles than previously recognized (Maherali & Klironomos, 2007).

Although root and soil AMF biomass responded overall differently to nutrient deficiencies, the response of individual AMF families and guilds were mostly consistent across both sample types (Tables **1**, **2**). N deficiency mostly affected edaphophilic (and partly ancestral) taxa, whereas P and K deficiencies mainly affected families within the rhizophilic guild, and to some extent, the ancestral guild in soil samples. This family-specific pattern may explain the distinct responses of AMF communities in roots and soil to specific nutrient deficiencies: total root AMF biomass, dominated by rhizophilic taxa was more susceptible to K deficiency – largely due to Glomeraceae, which comprised 87% of the community in roots , but strongly decreased under K deficiency (Fig. **2b**; Table **2**). In contrast, soil AMF biomass, dominated by edaphophilic and ancestral taxa, was strongly influenced by N and P (Fig. **2a**; Table **1**).

AMF Functional groups and families showed highly specific responses to nutrient deficiencies

The specific and significant responses of individual AMF families and guilds to nutrient deficiencies suggest that soil nutrient conditions exerted strong selection pressure on AMF functional traits, which are phylogenetically conserved at the family level. Although we grouped AMF families into functional guilds based on exploration traits (Weber *et al.*, 2019), other unknown functional traits may also be conserved at the family level, which may explain the different responses of some families within the same exploration guild.

While little is known about other potential functional traits of AMF at family levels, exploration traits have previously been used to explain family- and guild-level responses to nutrient conditions (Powell *et al.*, 2009; Treseder *et al.*, 2018; Weber *et al.*, 2019; Han *et al.*, 2020). Some reviews proposed that fungal taxa with high soil exploration capabilities, like Gigasporaceae, declined in N-enriched soils due to reduced C support from plants, even if this impaired the plant's ability to access nutrients such as phosphorus (Treseder *et al.*, 2018; Cotton, 2018; Lilleskov *et al.*, 2019). Contrastingly, fungi with limited soil exploration capabilities, such as Glomeraceae, might be favored in N-rich soils (Treseder *et al.*, 2018), maybe due to increased plant pathogen pressure at high nitrogenavailability. However, this conflicts with the trade-balance model, which proposes that plants prefer fungal partners with high soil exploration capacity under N-rich conditions to foster acquisition of other nutrients that could become limiting (Johnson, 2010). Aditionally, a global-scale meta-analysis has shown that N addition decreased the abundance of rhizophilic guilds (i.e. Glomeraceae) while minimally affecting edaphophilic guilds (i.e. Gigasporaceae) (Han *et al.*, 2020).

Our results support the findings of this meta-analysis, and oppose the concept that N fertilization reduces edaphophilic taxa. Although fewer AMF could thrive in N-fertilized soil, overall, the relative abundance of Gigasporaceae increased in both soil and roots. Similar to Han *et al.* (2020), we observed a negative effect of N addition on Glomeraceae, but evident only in root samples (Fig. **2**; Tables **1**, **2**) (Han *et al.*, 2020). Furthermore, we observed a significant positive correlation between the genus *Scutellospora* (which makes up about 99% of the Gigasporaceae) and inorganic N (ammonium and nitrate) as well as N:P ratio (Fig. **S16**), supporting the positive effect of N on Gigasporaceae.

Contrary to N, which mostly affected edaphophilic guilds, P and K primarily affected families within the rhizophilic guild (Fig. **2**; Tables **1**, **2**), with varying effects across families. While K deficiency strongly decreased Glomeraceae, P deficiency significantly decreased Paraglomeraceae in both soil and roots, and Claroideoglomeraceae in roots (Figs **2**, **S5**; Tables **1**, **2**). Correspondingly, Paraglomeraceae showed the strongest positive correlation with DOP out of all families in our dataset, (followed by Claroideoglomeraceae) (Fig. **S17**). Previous studies have revealed a positive link between P and Paraglomeraceae (Kurle & Pfleger, 1996; Hijri *et al.*, 2006), though this association was not observed by others (Gosling *et al.*, 2014). The negative impact of P deficiency on Paraglomeraceae contrasted with its positive effect on Ambisporaceae (ancestral guild), which significantly increased in P-deficient soils (Fig. **S6**; Tables **1**, **2**). Ambisporaceae were positively correlated with ammonium, nitrate and the N:P ratio, and negatively correlated with dissolved organic P (Fig. **S17**). Together our results hint

at contrasting functional roles between rhizophilic (especially Paraglomeracea) and ancestral (particularly Ambisporaceae) families regarding P availability. Interestingly, Ambisporaceae had the strongest positive response to P deficiency, surpassing even Gigasporaceae (Table **1**), a family that has traditionally received much more attention regarding soil nutrient limitation (Powell *et al.*, 2009; Treseder *et al.*, 2018). We thus hypothesize that Ambisporaceae might play a critical yet underappreciated role in P uptake from P-limited soils.

Similar to the response observed under P deficiency, K deficiency shifts the community from rhizophilic families to ancestral and, to some extent, edaphophilic families. K deficiency decreased the most abundant rhizophilic family, Glomeraceae, while significantly increasing Archaeosporaceae and Ambisporaceae (ancestral guild) and Diversisporaceae (edaphophilic guild) (Figs **S5**, **S6**, **S7**; Tables **1**, **2**). This pattern is most likely explained by plants leveraging the more extensive extraradical mycelia for P and K uptake under limiting conditions provided to a greater extent by ancestral and edaphophilic taxa. It is specifically noteworthy that families respond to specific nutrient deficiencies within each guild. While Ambisporaceae and Gigasporaceae may support plants under P deficiency, Archaeasporaceae and the Diversisporaceae seem to play a critical role under K deficiency. Notably, nitrogen deficiency uniquely increased Acaulosporaceae (ancestral guild). Overall, our analysis indicates that families with specific and complementary nutrient specializations exist within each of three guilds. Investigating their properties in more depth will pave the way to expand the concept of functional groups in AMF and to allow for a more targeted exploration of arbuscular mycorrhizal associations to reduce fertilizer use.

AMF were closely linked to plant communities and soil pH

AMF prefer neutral or alkaline soil pH (Helgason & Fitter, 2009; Han *et al.*, 2020), making soil pH a crucial factor for AMF abundance(Hu *et al.*, 2013; Avio *et al.*, 2020; Davison *et al.*, 2021; Riedo *et al.*, 2021). In our study, soil pH had the highest explanatory power for soil AMF biomass and community composition across all treatments (i.e. inorganic, organic and limed) (Figs **6c**, **S13**, **S14**). Although pH varied significantly (4.5-5.5) with inorganic treatments (excluding liming) (Figs **6c**, **S15a**; Table **S1**), it was less influential in explaining soil AMF biomass, which was mainly associated with inorganic and organic N and P concentrations (Figs **7**, **8a**). However, pH remained the best explanator for soil AMF community composition even in inorganic treatments (Figs **7**, **8c**).

Although AMF are not host-specific (Klironomos, 2000), they exhibit host preference (Vandenkoornhuyse *et al.*, 2002; Martínez-García *et al.*, 2015). Different functional plant

groups select distinct AMF communities, potentially based on fungal life-history traits (Chagnon *et al.*, 2013; Davison *et al.*, 2020; Blažková *et al.*, 2021). While these factors may partly explain the observed link between plant and soil AMF communities, plant and AMF communities may respond similar to changing soil parameters. Indeed, research demonstrated that soil conditions were more influential in shaping AMF communities in European grasslands than host plant specificity (Van Geel *et al.*, 2018). Edaphic factors may exert a stronger co-structuring effect on the soil-dominant AMF proportion, as extraradical hyphae are more exposed to edaphic parameters than intraradical hyphae. This could explain the stronger correlations between plant and soil AMF communities than between plant and root AMF communities (Fig. **5a,b**).

No effect of guild shifts and AMF biomass on soil C content

We also observed significant shifts between guilds across treatments, particularly between the fully fertilized plots (70% rhizophilic, 27% ancestral, 3% edaphophilic) and plots fertilized with N but deficient in P and K (24% rhizophilic, 65% ancestral, 10% edaphophilic; Fig. **S4**). AMF guilds are thought to affect soil C stocks through their mycelial structure and biomass (Horsch et al., 2023). Thus, we expected higher soil C contents in plots dominated by guilds with more extraradical mycelia (ancestral and edaphophilic) compared to rhizophilic guilds. However, while topsoil C content varied with nutrient deficiencies (Table **S1**), soil C did not correlate with AMF families and guilds (Fig. **S17**), AMF biomass, or plant biomass (Fig. **7**). The influence of nutrient deficiencies on soil C, despite no correlation with AMF or plants, suggests that soil C may be more affected by factors mediating C loss, like microbial organic matter decomposition, which is also influenced by nutrient deficiencies.

Conclusion

Our research indicates that specific and complementary nutrient specialisations exist at the family level within exploration-based guilds. Understanding AMF families' responses to nutrient limitations could benefit agricultural AMF inoculation strategies. Current practices prioritize a few model taxa, mostly from the Glomeraceae family (Lutz et al., 2023; Zhang et al., 2019). Including taxa with beneficial nutrient specialisation traits could enhance these strategies. Our results also emphasize the importance of analyzing the whole spectrum of AMF families, which was made possible in our study by sequencing the 18S rRNA gene with AMF-specific primers. While past AMF research has traditionally been biased towards Glomeraceae due to ITS-based sequencing approaches, our research clearly demonstrates the need for a better understanding on other AMF families, specifically ancestral ones.

Overall, our research shows that 70 years of biomass removal resulted in N, P, and K deficiencies and imbalances, significantly altering AMF communities and affecting ecosystem functions. Prolonged K deficiency, a relatively understudied area in mycorrhizal research, significantly affected AMF colonization of plant roots and the plant-to-soil AMF biomass ratio. This study emphasizes the necessity of long-term nutrient balance to support healthy agro-ecosystems and optimal plant-AMF interactions.

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Competing interests

None declared

Author contributions

KJ: Conceptualization, Formal Analysis, Investigation, Visualization; **LA:** Formal Analysis; **KG:** Formal Analysis, Visualization, Methodology; **SG:** Formal Analysis, Investigation, Visualization; **SD:** Investigation; **LF:** Formal Analysis, Investigation; **AC:** Formal Analysis, Investigation; **VM:** Investigation; **JW:** Investigation; **FS:** Investigation; **BI:** Investigation; **HS:** Investigation; **KH:** Conceptualization; **EMP:** Funding Acquisition, Project Administration, Resources; **AR:** Conceptualization, Funding Acquisition, Project Administration, Supervision, Resources; **JJ:** Conceptualization, Formal Analysis, Investigation, Methodology, Supervision; **CK:** Conceptualization, Funding Acquisition, Project Administration, Supervision, Validation, **KJ** and **CK** wrote the original draft and all other co-authors contributed to review and editing.

Data availability

Sequencing data are available through NCBI - GenBank under accession number PRJNA1140020. All other data can be found in a public repository: https://doi.org/10.5281/zenodo.13760726

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Supporting Information

Fig. S1 Experimental plot setup.

Fig. S2 Relative abundance of soil AMF community composition based on RNA and DNA amplicon sequencing of 18S rRNA gene.

Fig. S3 The effects of long-term nutrient deficiencies on soil (DNA-based) and root (DNA-based) AMF communities based on correspondence analysis (CA).

Fig. S4 The Effects of long-term deficiencies of N, P and K on soil and root AMF functional guilds.

Fig. S5 Effects of long-term nutrient deficiencies on soil and root AMF rhizophilic guild.

Fig. S6 Effects of long-term nutrient deficiencies on soil and root AMF ancestral guild.

Fig. S7 Effects of long-term nutrient deficiencies on soil and root AMF edaphophilic guild.

Fig. S8 The effects of long-term nutrient deficiencies on soil (RNA-based) and root (DNA-based) AMF community composition.

Fig. S9 The impact of long-term nutrient deficiencies on soil (DNA-based) AMF community composition.

Fig. S10 The influence of long-term nutrient deficiencies on soil AMF communities (DNA-based) across all treatments.

Fig. S11 The impacts of long-term nutrient deficiencies on plant community composition.

Fig. S12 Long-term nutrient deficiencies effects on plant communities across all treatments.

Fig. S13 Relationship between AMF and environmental parameters across all treatments.

Fig. S14 Independent and joint contribution of different factors to R^2 in soil and root AMF.

Fig. S15 The impact of long-term nutrient deficiencies on the soil pH and soil water content.

Fig. S16 Correlations between AMF genera and environmental factors.

Fig. S17 Correlations between AMF functional traits as well as families, and environmental factors.

Fig. S18 Effects of long-term organic fertilization (Manure and slurry) on soil and root AMF biomass.

Table S1 Three-way ANOVA results regarding the effect of long-term deficiencies of N, P and K on edaphic factors in inorganic treatments

Notes S1 Evaluating the influence of environmental drivers on soil and root AM fungal biomass and community composition.

Table 1. Effects of long-term N, P and K deficiencies on soil AMF biomass, families and guilds in inorganic treatments .

Treatment	Relative abundance	-N	-P	-K	-N*-P	-N*-K	-P*-K	-N*-P*-K
Soil AMF biomass		**0.000***↑**	0.094 · ↓	0.310	0.935	**0.005****	0.088 ·	0.357
Soil AMF biomass per plant biomass unit		**0.000***↑**	**0.005**↑**	0.170	0.149	**0.575**	**0.002****	0.182
Rhizophilic guild	0.52 ± 0.03	0.653	**0.000***↓**	**0.040*↓**	**0.027***	0.990	0.161	0.201
Glomeraceae	0.34 ± 0.03	0.146	0.830	**0.009**↓**	0.825	0.499	0.053 ·	0.554
Claroideoglomeraceae	0.05 ± 0.01	0.442	0.150	**0.033*↑**	0.591	0.071 ·	0.333	0.324
Paraglomeraceae	0.13 ± 0.03	0.255	**0.000***↓**	0.523	**0.037***	0.795	0.441	0.395
Edaphophilic guild	0.07 ± 0.01	0.358	0.327	0.429	0.431	0.817	0.252	0.296
Diversisporaceae	0.02 ± 0.01	0.091 · ↑	0.238	**0.007**↑**	0.673	0.114	0.476	0.488
Gigasporaceae	0.05 ± 0.01	**0.046*↓**	0.071 · ↑	0.579	0.231	0.571	0.288	0.345
Ancestral guild	0.41 ± 0.03	0.964	**0.000***↑**	0.106	0.076 ·	0.895	0.057 ·	0.082 ·
Archaeosporaceae	0.04 ± 0.01	0.080 · ↓	0.148	**0.000***↑**	**0.035***	0.169	**0.032***	0.178
Acaulosporaceae	0.23 ± 0.02	**0.001**↑**	0.388	0.382	0.434	0.061 ·	0.353	0.991
Ambisporaceae	0.14 ± 0.02	**0.000***↓**	**0.000***↑**	**0.010*↑**	**0.002****	**0.015***	0.210	**0.016***

*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, · : $P < 0.1$, ↑: positive main effect, ↓: negative main effect

Shown are P values from a three-way ANOVA using N, P and K deficiency (-N,-P,-K) as factors, including testing their interactions (-N*-P, -N*-K,-P*-K,-N*-P*-K) . The analysis is based on a fully factorial design of N, P and K deficiency with 4 replicate plots per treatment (n=4, total nr of plots: 32). Relative abundances of guilds and families are presented as the mean across all inorganic treatments (+/-standard error, n= 32). Bold values show *P* below 0.05 and the nutrients in header represent deficiency.

Table 2. Three-way ANOVA results regarding the impact of long-term N, P and K deficiencies on root AMF biomass as well as families and guilds in inorganic treatments

Treatment	Relative abundance	-N	-P	-K	-N*-P	-N*-K	-P*-K	-N*-P*-K
Root AMF biomass		0.054 · ↑	0.661	**0.001****↓	0.841	0.146	0.672	0.978
Rhizophilic guild	0.91 ± 0.02	0.143	0.505	**0.000*****↓	0.100	0.610	0.523	0.563
Glomeraceae	0.87 ± 0.02	**0.019***↑	0.456	**0.000*****↓	0.101	0.828	0.805	0.376
Claroideoglomeraceae	0.03 ± 0.00	0.058 · ↓	**0.000*****↓	**0.001****↑	0.417	**0.041***	0.171	0.762
Paraglomeraceae	0.01 ± 0.00	**0.008****↓	**0.000*****↓	**0.026***↑	**0.003****	0.198	0.260	0.098 ·
Edaphophilic guild	0.00 ± 0.00	**0.006****↓	0.845	**0.012***↑	0.513	0.098 ·	0.901	0.236
Diversisporaceae	0.00 ± 0.00	0.917	0.434	0.086 · ↑	0.335	0.638	0.922	0.118
Gigasporaceae	0.00 ± 0.00	**0.000*****↓	0.788	0.144	0.791	0.277	0.749	0.445
Ancestral guild	0.09 ± 0.02	0.255	0.542	**0.000*****↑	0.139	0.661	0.401	0.931
Archaeosporaceae	0.01 ± 0.00	0.566	0.135	**0.000*****↑	0.150	0.657	0.974	0.469
Acaulosporaceae	0.01 ± 0.00	**0.004****↑	0.816	0.811	0.781	0.722	0.894	0.988
Ambisporaceae	0.07 ± 0.02	0.072 · ↓	0.464	**0.002****↑	0.326	0.976	0.562	0.652

*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, · : $P < 0.1$, ↑: positive main effect, ↓: negative main effect

The table illustrates P values obtained from a three-way ANOVA using N, P and K deficiency (-N,-P,-K) as factors, including testing their interactions (-N*-P, -N*-K,-P*-K,-N*-P*-K) . The analysis is derived from a fully factorial design of N, P and K deficiency with 4 replicate plots per treatment (n=4, total nr of plots: 32). Relative abundances of guilds and families are presented as the mean across all inorganic treatments (+/-standard error, n= 32). Bold values display *P* below 0.05 and the nutrients in header indicate deficiency.

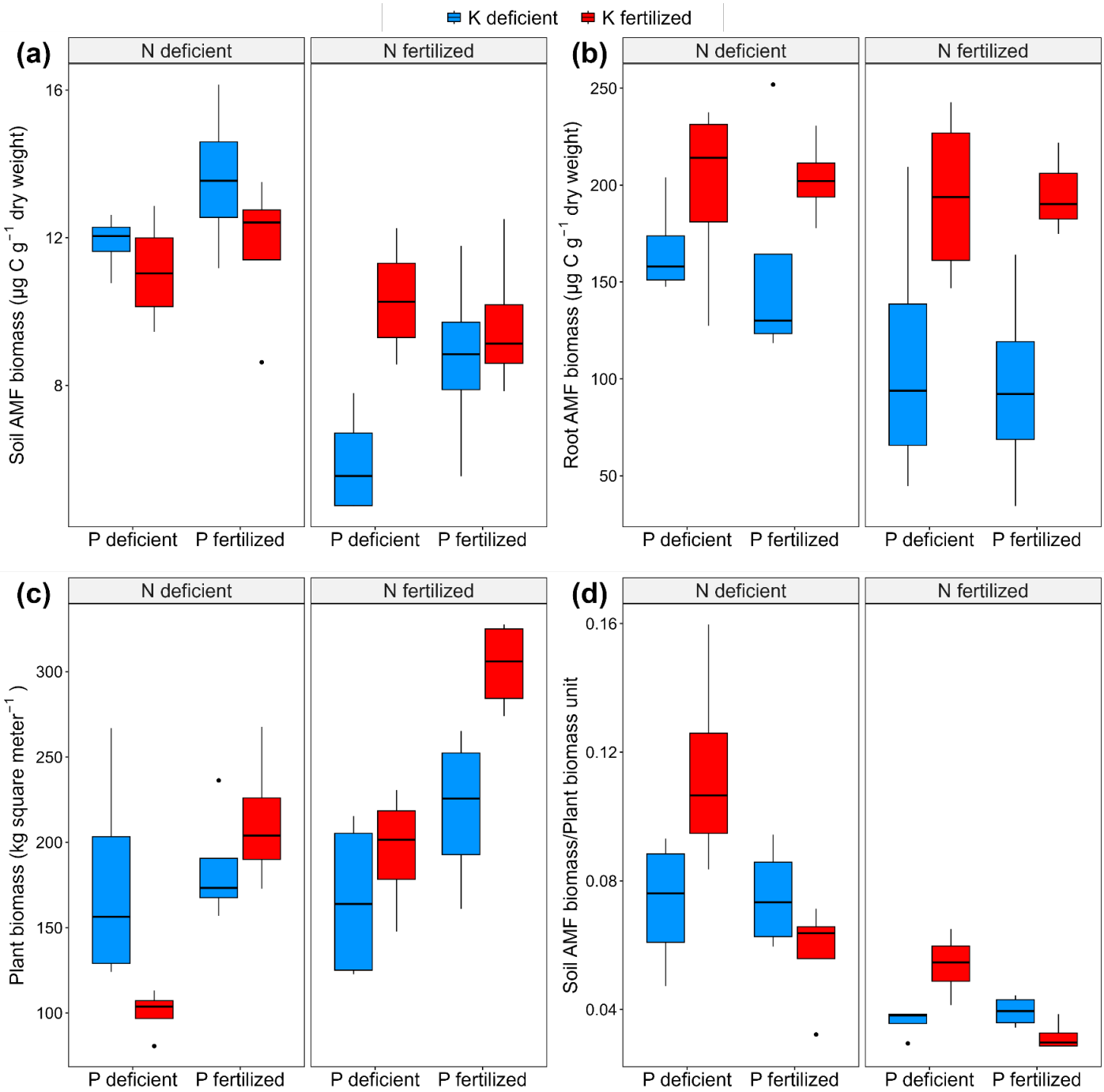

Figure 1. Effects of long-term nutrient deficiencies on soil and root AMF, and plant biomass. N deficiency significantly increased soil AMF biomass, while K deficiency significantly reduced root AMF biomass (p<0.05, ANOVA, see Tables **1**, **2**). The y-axes in the boxplot shows a) soil (i.e. extraradical) and b) root (i.e. intraradical) AMF biomass quantified using 16:1ω5 fatty acid in neutral lipid fraction, as well as, c) aboveground plant biomass and d) soil AMF biomass per single plant biomass unit. Each figure depicts N deficiency (left panels) and N fertilization (right panels). The x-axes show P availability, and the blue (deficient) and red (fertilized) colors are related to K in each panel. Boxplots indicate the first and third quartiles and the solid lines represent the median (n=4).

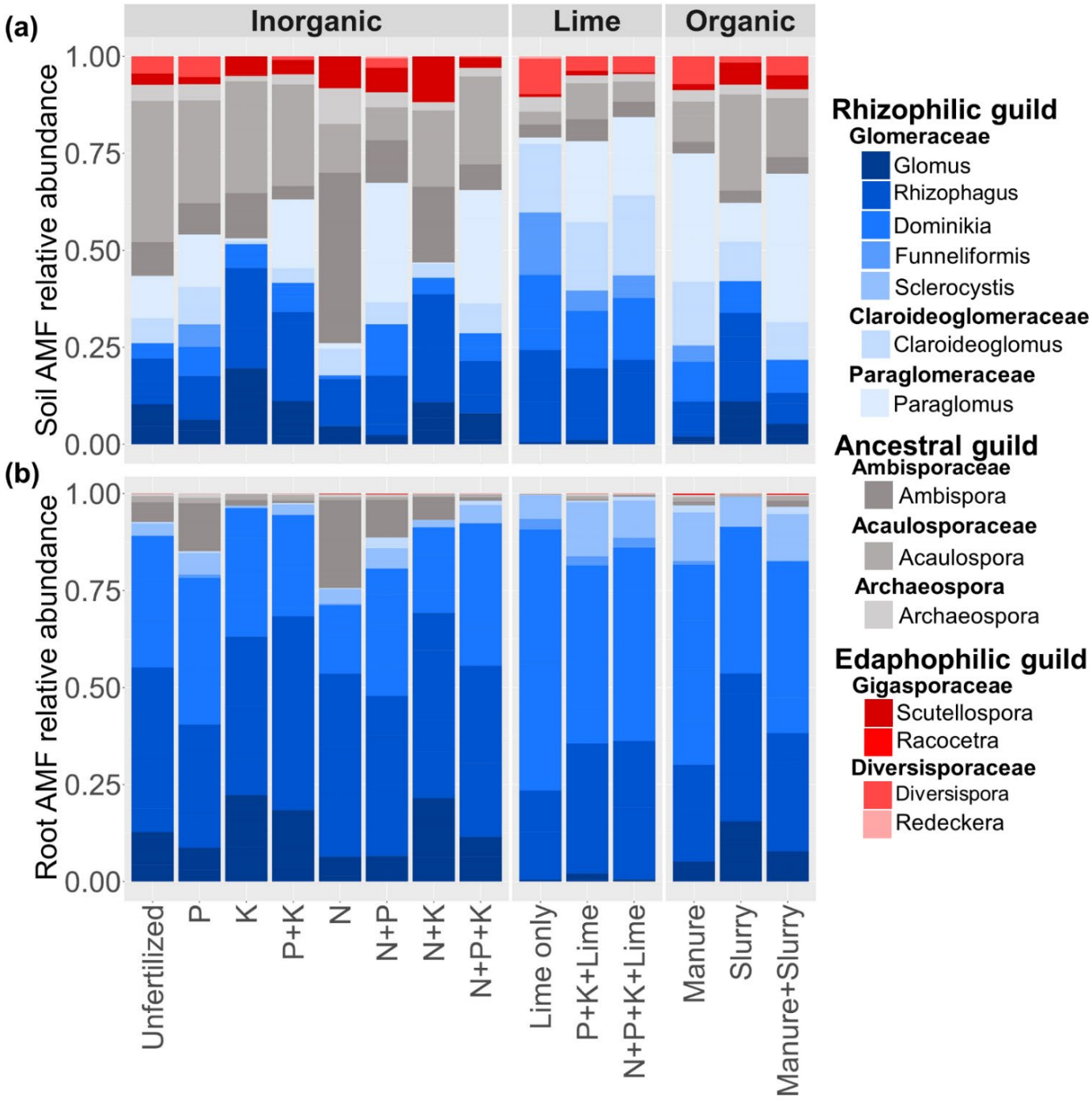

Figure 2. Impact of long-term nutrient deficiencies on soil and root AMF community composition. Long-term nutrient deficiencies and treatments uniquely influenced AMF communities in soil and roots (p<0.05, ANOVA, see the Tables **1**, **2**). Panels (a) and (b) depict relative abundances of soil and root AMF genera as stacked bar plot and it is based on RNA (soil samples) and DNA (root samples) amplicon sequencing of 18S rRNA gene. The relative abundances of rhizophilic, ancestral, and edaphophilic guilds are depicted in blue, grey, and red, respectively. AMF community composition was analysed by RNA (soil) and DNA (root) amplicon sequencing of the 18S rRNA gene.

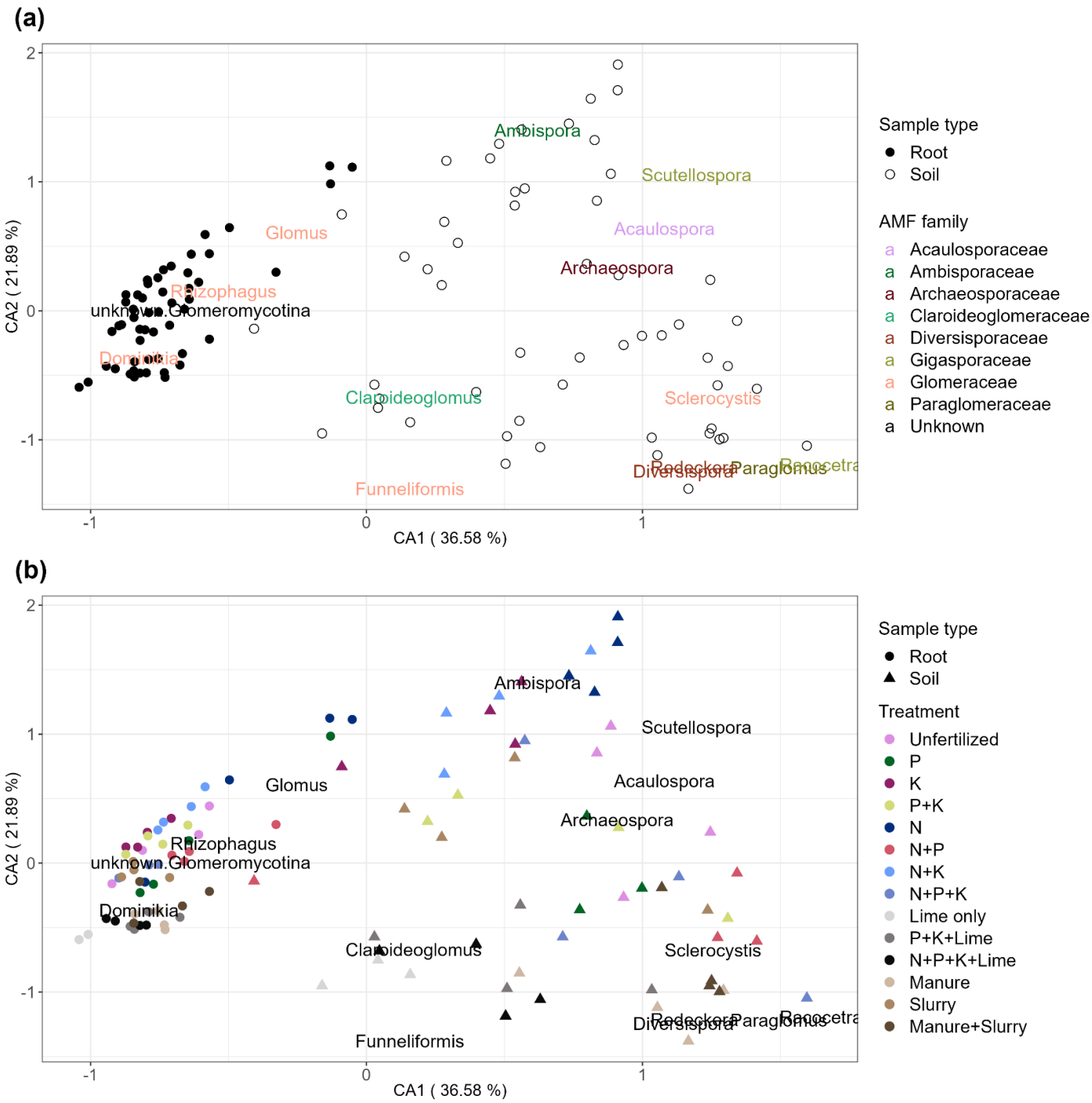

Figure 3. Compositions of AMF communities in soil and root samples exposed to different long-term nutrient deficiencies evaluated by correspondence analysis (CA). Communities were most distinct between soil and root samples (separation along the first CA axis); within each sample type (root or soil) they were significantly separated by treatments (along the second CA axis) (PERMANOVA, $p<0.001$). Soil and root AMF community compositions were assessed by RNA and DNA amplicon sequencing of 18S rRNA gene, respectively (for a comparison of soil DNA and root DNA data see fig. **S3**). In panel a), the shapes demonstrate the soil (○) and root (●) samples, and the colors indicate the different AMF orders. Panel b shows different fertilization treatments by colors and the shapes indicate soil (▲) and root (●) samples. The text labels represent AMF genera.

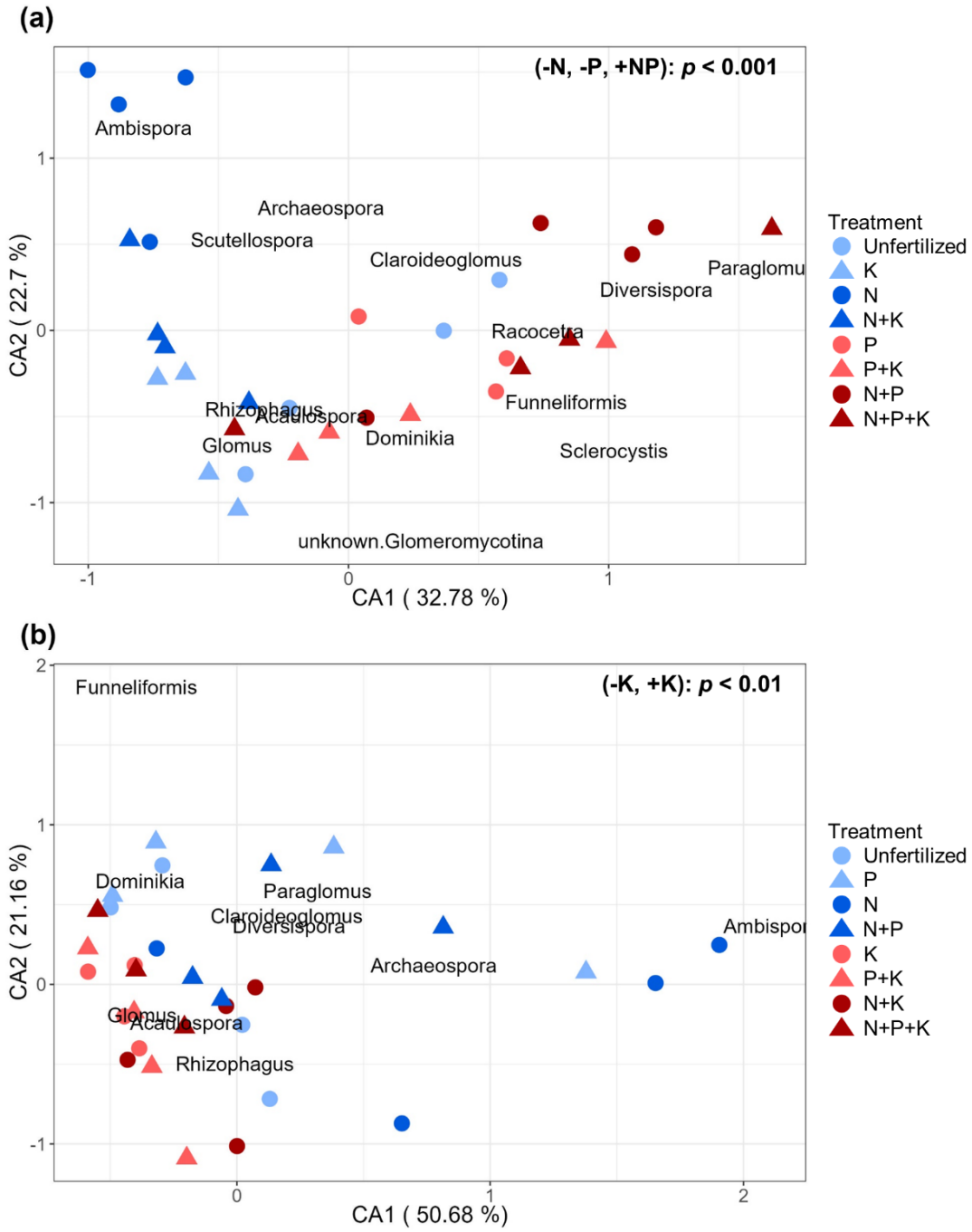

Figure 4. The effects of long-term nutrient deficiencies on soil and root AMF community composition based on correspondence analysis (CA). The soil AMF community composition was significantly affected by N and P treatments (PERMANOVA for P deficient, N deficient and P plus N fertilized treatments: F=6.53, P<0. 001), whereas the root AMF community composition was influenced by K (PERMANOVA for K deficient and K fertilized treatments: F=5.23, P<0. 01). The biplot illustrates only the effects of inorganic treatments on soil (a) and root (b) AMF community composition and it is calculated based on CA. In soil samples (a), the blue and red colors indicate P deficient and fertilized plots, respectively, whereas, in roots (b), The blue and red colors show K deficient and fertilized plots, respectively. The shapes show K deficient (●) and fertilized (▲) treatments in soil samples (a) and P deficient (●) and fertilized (▲) in roots (b). Light and dark colors are related to N deficient and fertilized treatments, sequentially, in both samples and the text labels are indicators of AMF genera. For a correspondence analysis of all treatments please see supplementary Fig. **S8**.

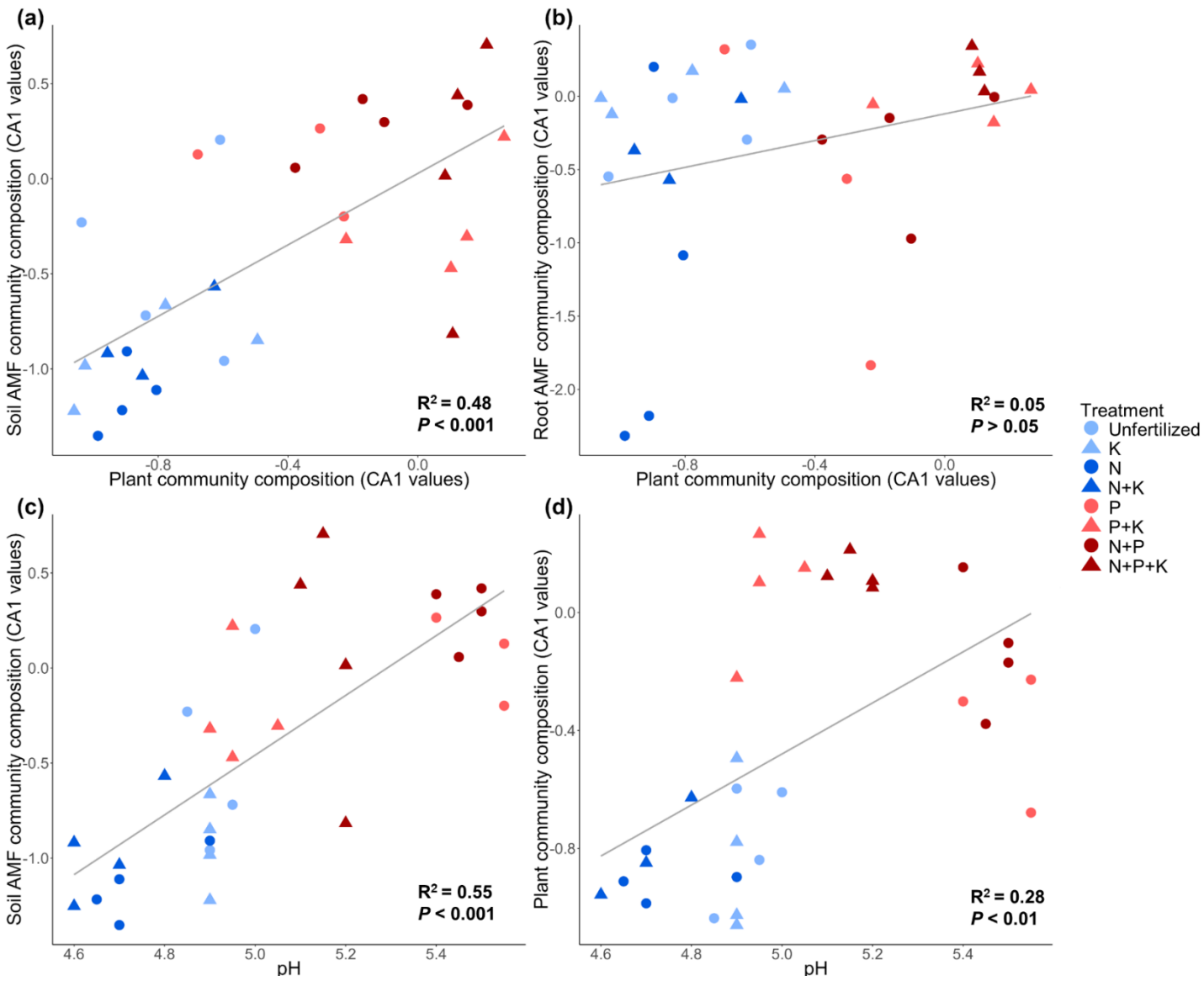

Figure 5. Correlations among plant, AMF community compositions, and pH. Plant community composition was strongly correlated with soil AMF community composition and both soil AMF and plant community compositions had significant correlation with pH. The scatterplots represent the correlation between plant community composition, and a) soil and b) root AMF community compositions as well as the relationships of both c) soil AMF and d) plant community composition with pH in inorganic treatments. As a proxy for community compositions, the CA1 scores of correspondence analyses were used. The blue and red colors show P deficient and fertilized plots, respectively. The shapes show K deficient (●) and fertilized (▲) treatments. Light and dark colors are associated with N deficient and fertilized treatments, sequentially.

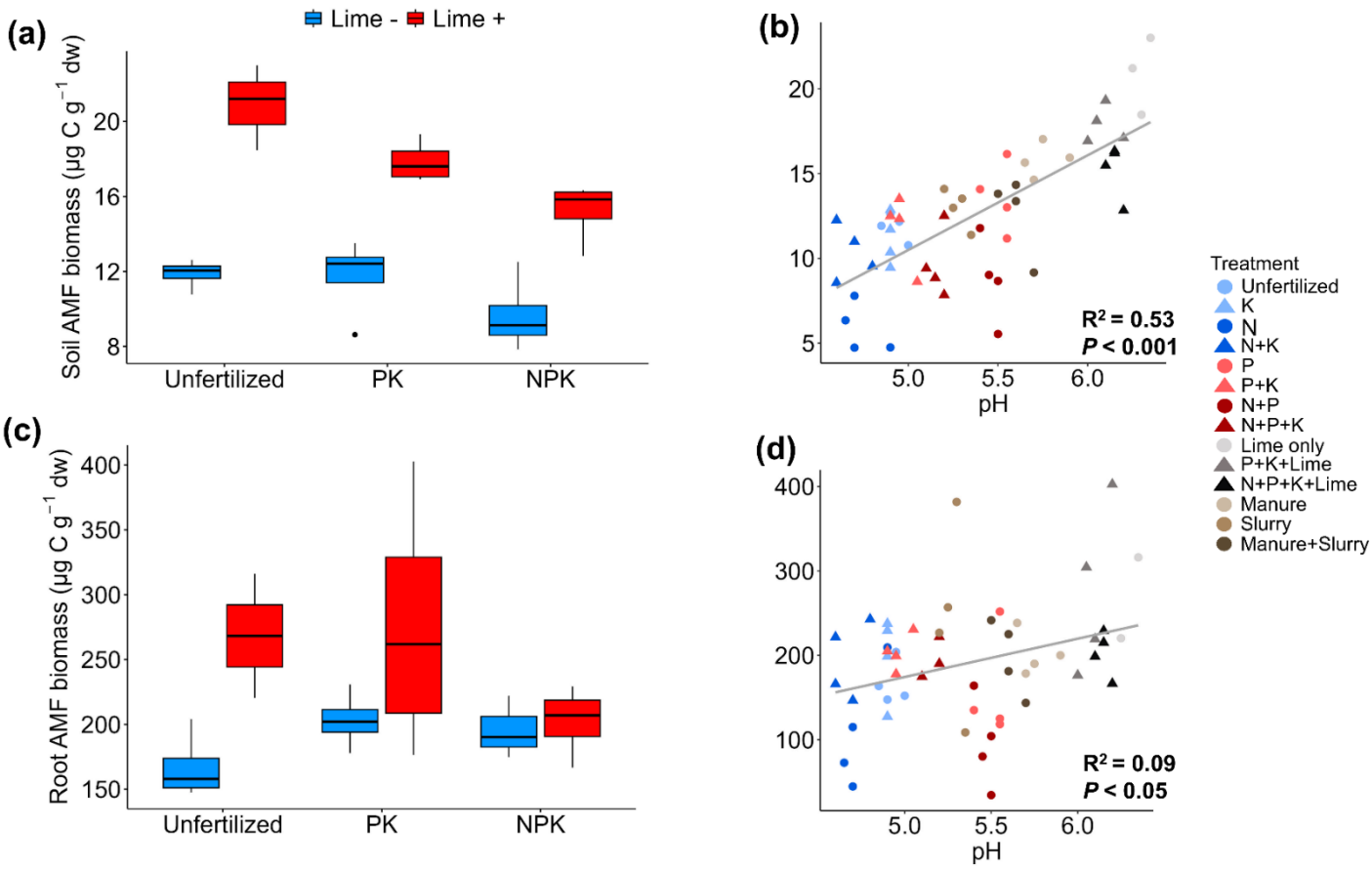

Figure 6. The impact of long-term lime application and fertilization regiment on soil and root AMF biomass as well as the relationship of soil and root AMF biomass with pH. Lime significantly increased AMF biomass but had stronger a stronger impact on Soil AMF biomass compared to root AMF biomass. Furthermore, the correlation between pH and soil AMF biomass was stronger Relative to root AMF biomass. The y-axes in boxplots represent a) soil and c) root AMF biomass measured using 16:1ω5 fatty acid in neutral lipid fraction. Boxplots indicate the first and third quartiles and the solid lines represent the median (n=4) and the colors are associcated with presence (red) and absence (blue) of Lime. X-axes in boxplots indicate fertilization regiment. The scatterplots illustrate the correlation of b) soil and d) root AMF biomass with pH. In scatterplots, the blue and red colors represent P deficient and fertilized plots, respectively. The shapes indicate K deficient (●) and fertilized (▲) plots. Light and dark colors illustrate N deficient and fertilized treatments, respectively.

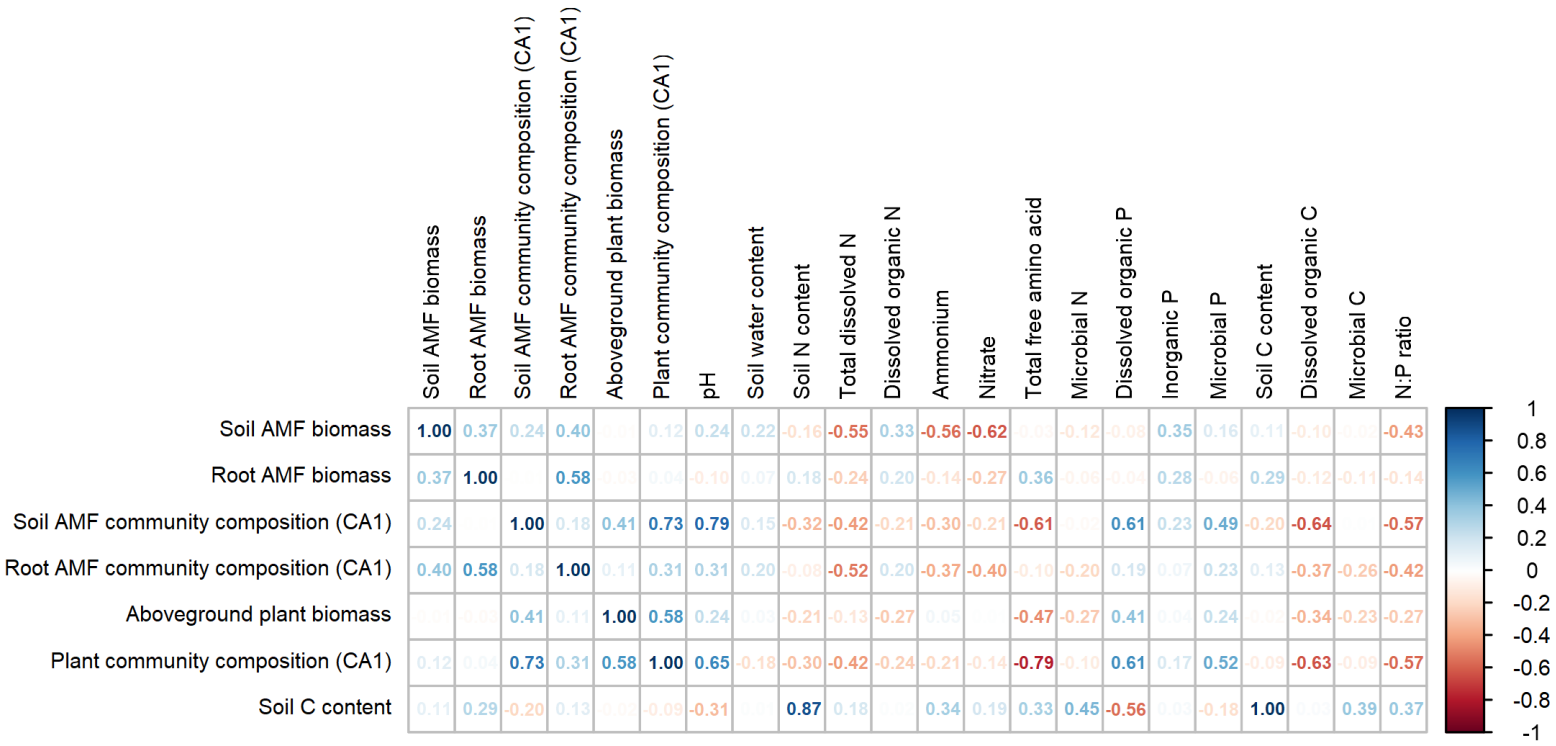

Figure 7. Relationships between AMF, plants and environmental parameters in inorganic treatments. Numbers show pairwise Spearman's correlation coefficients between AMF biomass and community composition, plant biomass and community composition, and various soil parameters. Blue colors indicate positive and red colors negative correlations. Correlation analysis was based on inorganic treatments only. For correlation analyses among all treatments please see supplementary Fig. **S13**.

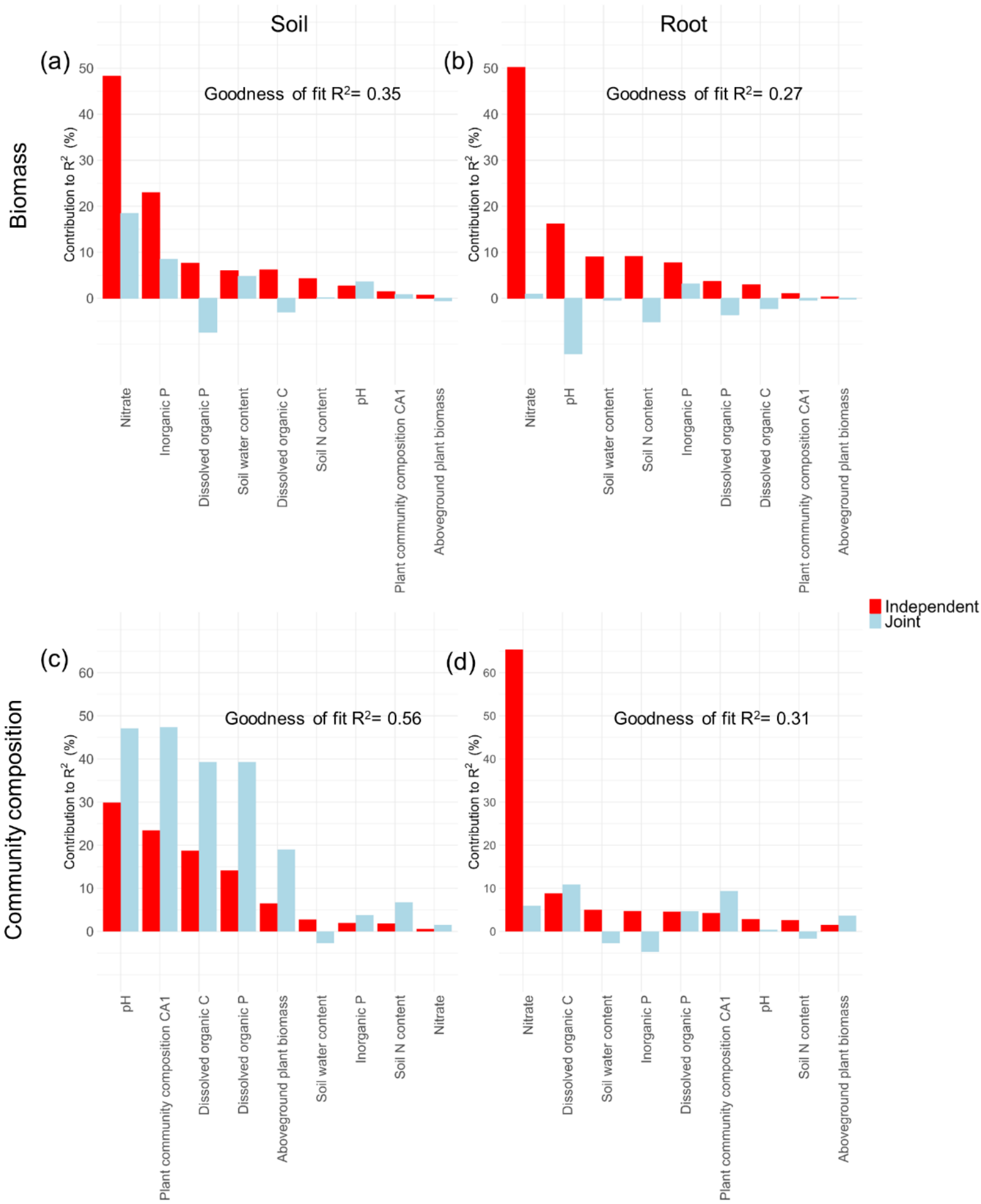
Biomass
Soil
Root
(a)
(b)
Goodness of fit R²= 0.35
Goodness of fit R²= 0.27
Contribution to R² (%)
Nitrate
Inorganic P
Dissolved organic P
Soil water content
Dissolved organic C
Soil N content
pH
Plant community composition CA1
Aboveground plant biomass
Independent
Joint
Community composition
(c)
(d)
Goodness of fit R²= 0.56
Goodness of fit R²= 0.31

Figure 8. Ranking of factors explaining variability in soil and root AMF abundance and community compositions across inorganic fertilisation treatments. Nitrate concentrations explained the highest proportion of the variability in AMF biomass, and root community composition, while pH and plant community composition were linked most strongly to soil AMF community composition. Shown are the results of a hierarchical variance partitioning. Plots show the contribution of various soil and plant parameters to the goodness of fit (R2) of a hierarchical variance partitioning analysis for soil and root AMF abundance (a,b) and community composition (c,d,) . Independent effects are shown by blue bars while joint effects are vizualized by red bars. For results of hierarchical partitioning across all treatments please see supplementary Fig. **S16**.