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Grassland ecosystem type drives AM fungal diversity and functional guild distribution in North American grasslands

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Abstract

Nutrient exchange forms the basis of the ancient symbiotic relationship that occurs between most land plants and arbuscular mycorrhizal (AM) fungi. Plants provide carbon (C) to AM fungi and fungi provide the plant with nutrients such as nitrogen (N) and phosphorous (P). Nutrient addition can alter this symbiotic coupling in key ways, such as reducing AM fungal root colonization and changing the AM fungal community composition. However, environmental parameters that differentiate ecosystems and drive plant distribution patterns (e.g., pH, moisture), are also known to impact AM fungal communities. Identifying the relative contribution of environmental factors impacting AM fungal distribution patterns is important for predicting biogeochemical cycling patterns and plant-microbe relationships across ecosystems. To evaluate the relative impacts of local environmental conditions and long-term nutrient addition on AM fungal abundance and composition across grasslands, we studied experimental plots amended for 10 years with N, P, or N and P fertilizer in different grassland ecosystem types, including tallgrass prairie, montane, shortgrass prairie, and desert grasslands. Contrary to our hypothesis, we found ecosystem type, not nutrient treatment, was the main driver of AM fungal root colonization, diversity, and community composition, even when accounting for site-specific nutrient limitations. We identified several important environmental drivers of grassland ecosystem AM fungal distribution patterns, including aridity, mean annual temperature, root moisture, and soil pH. This work provides empirical evidence for niche partitioning strategies of AM fungal functional guilds and emphasizes the importance of long-term, large scale research projects to provide ecologically relevant context to nutrient addition studies.

KEYWORDS
community ecology, ecological genetics, fungi, microbial ecology, mycorrhizae, nutrient network

1 | INTRODUCTION

Mycorrhizal fungi serve as a critical link in the plant–soil continuum (Wilson et al., 2009) and facilitate the flow of nutrients from the soil to plants. In a bidirectional exchange, plants exude surplus photosynthate from roots and arbuscular mycorrhizal (AM) fungi supply the plant with soil nutrients, often referred to as a C for nutrient exchange (Werner & Kiers, 2015), or more recently in the framework of surplus C allocation (Prescott et al., 2020). Nutrient additions have been shown to reduce the level of arbuscular mycorrhizal (AM) fungal colonization in roots (Blanke et al., 2005), reduce the abundance of AM fungi in soil (Carrara et al., 2018; Frater et al., 2018; Leff

et al., 2015), and/or alter the community composition of AM fungi in roots and soil (Phillips et al., 2019). This is because nitrogen (N) and/or phosphorus (P) addition can lead to reduced allocation in belowground carbon (C) from plants (Bever et al., 2009; Ji & Bever, 2016; Werner & Kiers, 2015).

However, nutrient additions can both promote (Treseder & Allen, 2002) and suppress (Ma et al., 2020) AM fungal abundance and diversity. It has been suggested that the differential AM fungal responses to nutrient additions are related to the local availability of nutrients (Johnson, 2010; Johnson et al., 2010). In nutrient limited sites, both N and P fertilization have been shown to increase AM fungal biomass and diversity instead of having the negative outcome one might traditionally expect (Cheng et al., 2013; Egerton-Warburton et al., 2007; Treseder & Allen, 2002). Because there are competing theories on how plants and AM fungi respond to nutrient additions, it remains difficult to predict how AM fungi will respond to long-term fertilization under varying field conditions.

In addition to nutrient availability, other environmental characteristics have been identified as important drivers of soil microbial distribution patterns, including AM fungi. For example, mean annual temperature and pH were positively correlated with AM fungal root colonization in the native tallgrass prairie species *Schizachyrium scoparium* (Frater et al., 2018). Studies on the impact of drought and flooding on AM fungi have also identified moisture as an important driver of AM fungal diversity, root colonization, and community composition (Deepika & Kothamasi, 2015; Weber et al., 2019). While nutrient amendments and other environmental factors impact AM fungal community dynamics in parallel, a cohesive understanding of the relative impacts of environmental characteristics and nutrient availability on AM fungal distribution patterns remains elusive.

Grassland ecosystems, where many late successional native plant species are highly dependent on symbiotic interactions with AM fungi (Cheeke et al., 2019), are especially influenced by AM fungal associations. Although AM fungi have been more extensively studied in temperate grasslands compared to any other biome (Powell & Rillig, 2018), "temperate grassland" is a broad classification that encompasses many different ecosystem types (e.g., tallgrass prairies, shortgrass prairies) with distinct plant and fungal communities that may respond differently to perturbations, such as nutrient addition. The C for nutrient exchange dynamics between plant hosts and AM fungi have been well described in highly controlled systems (laboratory, greenhouse, agricultural) (Bever et al., 2009; Jones & French, 2021; Williams et al., 2017) and in field experiments focused on one or two plant species (Frater et al., 2018; Klichowska et al., 2019; Pan et al., 2020; Xu et al., 2017). Yet monoculture or species-specific responses are not readily scaled to diverse grasslands. Due to their importance in plant- nutrient cycling and the differential nutrient distribution patterns between AM fungal taxa (Sikes et al., 2010), an ecosystem-scale understanding of the drivers of AM fungal distribution patterns is needed.

Integrating the diversity of AM fungal responses into a generalized conceptual model requires using trait-based approaches that cluster taxonomic groups into functional categories (Crowther et al., 2014). A description of AM fungi functional guilds based on taxonomy assignments by Weber et al. (2019) enables targeted investigations into AM fungal distribution patterns. Three functional guilds of AM fungi have been defined at the family level by the preferential distribution of hyphae into the root (intraradical hyphae) or outside of the root (extraradical hyphae; Weber et al., 2019). Rhizophilic AM fungi (e.g., Glomeraceae, Claroideoglomeraceae, Paraglomeraceae; Weber et al., 2019) allocate proportionally more of their C to intraradical hyphae and are thought to help protect against plant pathogens (Sikes et al., 2010). Edaphophilic AM fungi (e.g., Gigasporaceae, Diversisporaceae) allocate more of their C to extraradical hyphae and may increase the host plant's ability to obtain nutrients and moisture (Finlay, 2008). Finally, ancestral AM fungi (e.g., Archaeosporaceae, Ambisporaceae, Pacisporaceae, Acaulosporaceae) do not appear to preferentially allocate hyphae to either roots or soil (Weber et al., 2019). Supporting extensive extraradical hyphal networks in soil comes at a C cost to the plant and it has been suggested that plants may select against edaphophilic fungal species when soil nutrients are readily available (Treseder et al., 2018). These guild-specific traits provide a means of scaling from specific plant-microbe interactions to a community- or ecosystem-scale understanding of AM fungal responses to different environmental perturbations, like nutrient amendments or drought.

To compare the influence of environmental characteristics and long-term (>10 years) nutrient addition (N, P, N+P) on AM fungal community composition, diversity, and the relative abundance of AM functional guilds across grasslands, we selected a broad (geographic, ecological) set of grassland sites across the United States to test two main hypotheses. First, we hypothesized that environmental factors (e.g., pH, moisture) characteristic to each grassland ecosystem type would be associated with variation in AM fungal community structure, diversity, and functional guild distribution across sites. Second, we hypothesized that soil nutrient amendments would cause plants to reduce C allocation to AM fungi, selecting against edaphophilic AM fungi in nutrient amended plots because of the unnecessary cost of supporting extensive extraradical hyphal networks for nutrient acquisition.

2 | MATERIALS AND METHODS

2.1 | Study sites

Soil and root samples were collected from eight long-term ecological research (LTER) sites across four different grassland ecosystem types (montane, tallgrass, shortgrass, and desert) located throughout the United States, varying in climate, soil texture, and other environmental parameters (Table 1). The sites were located in Bunchgrass Andrews LTER, Oregon (BNCH); Cedar Point Biological Station, Nebraska (CDPT); Cedar Creek LTER, Minnesota

TABLE 1 Ecosystem and soil properties from control plots for each site to provide a baseline of the soil and nutrient differences between each site

Site name	Lookout ridge, OR ($n = 3$)	Bunchgrass, OR ($n = 3$)	Sevilleta LTER, NM ($n = 4$)	Cedar Creek LTER, MN ($n = 4$)	Shortgrass steppe LTER, CO ($n = 3$)	Cedar point Biological Station, NE ($n = 4$)	Trelease LTER, CO ($n = 3$)	Konza LTER, CO ($n = 3$)
Abb.	LOOK	BNCH	SEV	CDR	SGS	CDPT	TREL	KZA
Ecosystem type	Montane grassland	Montane grassland	Desert grassland	Tallgrass prairie	Shortgrass prairie	Shortgrass prairie	Tallgrass prairie	Tallgrass prairie
Latitude (°)	44.2	44.3	34.4	45.4	40.8	41.2	40.1	39.1
Longitude (°)	−122.1	−122	−106.7	−93.2	−104.8	−101.6	−88.8	−96.6
Elev. (m)	1500	1318	1600	270	1650	965	200	440
MAP (mm)	898	647	52	50	65	45	992	889
MAT (°C)	4.8	5.5	12.6	6.3	8.4	9.5	11.1	12.1
Aridity Index	2.31	1.93	0.17	0.84	0.32	0.4	0.89	0.76
N deposition ($kg\,ha^{-1}\,year^{-1}$)	2.84	2.84	1.96	6.98	3.12	3.12	16.79	9.83
pH	5.3 (0.02)	5.5 (0.05)	7.3 (0.15)	5.9 (0.30)	6.8 (0.44)	6.4 (0.08)	6.3 (0.46)	6.1 (0.03)
Soil C (%)	18.63 (0.64)	7.86 (0.56)	0.31 (0.03)	0.58 (0.11)	0.83 (0.06)	1.19 (0.09)	3.42 (0.12)	4.49 (0.26)
Soil N (%)	1.26 (0.06)	0.54 (0.06)	0.03 (0.001)	0.04 (0.006)	0.08 (0.001)	0.1 (0.006)	0.27 (0.007)	0.32 (0.01)
Soil P (ppm)	63.3 (7.2)	13 (1.0)	37.25 (3.64)	59 (2.1)	68.5 (5.5)	33.25 (2.75)	54 (1.5)	14 (4.0)
Clay (%)	0.8	2.9	5.6	2.5	8.8	9.8	15.6	n/a
Silt (%)	30.1	26.5	9.9	8.1	17.1	19.9	64.1	n/a
Sand (%)	69	70.4	84.4	89.3	74	70.2	20.2	n/a

Note: Values are means (standard errors) of three or four replicated blocks from the same site.

Abbreviations: MAP, mean annual precipitation; MAT, mean annual temperature; soil pH, moisture, and texture from Nutrient Network collaborators.

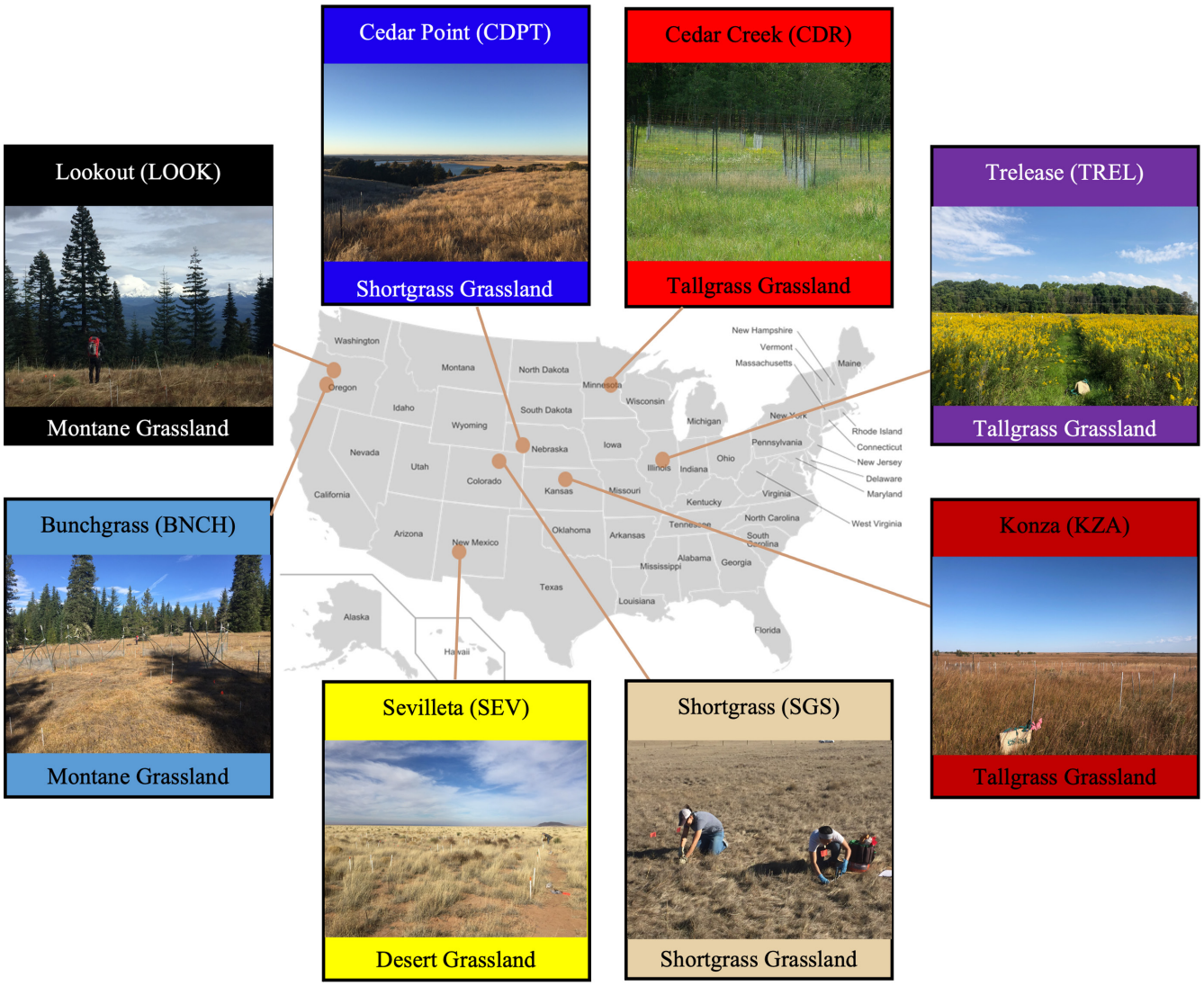

FIGURE 1 Map of sample locations with site names, abbreviations, and ecosystem type. [Colour figure can be viewed at wileyonlinelibrary.com]

(CDR); Konza Prairie LTER, Kansas (KZA); Lookout Andrews LTER, Oregon (LOOK); Sevilleta LTER, New Mexico (SEV); Shortgrass Steppe LTER, Colorado (SGS); Trelease, Illinois (TREL) (Figure 1). All sites are part of the Nutrient Network (NutNet) project (https://nutnet.org/), a global ecosystem-scale nutrient addition field experiment replicated at over 100 grassland sites in 25 countries on five continents (Borer et al., 2014). At each site, the experimental design includes replicated fertilized and unfertilized 5 × 5 m experimental plots for the investigation of biological responses to nutrient amendments and herbivory (Borer et al., 2014). We focused on a subset of nutrient addition plots in the full NutNet treatment design, including N (+N; 10 g N m^{-2} $year^{-1}$ as time-released urea), P (+P; 10 g P m^{-2} $year^{-1}$ as triplicate super phosphate), N–P (+NP; each at 10 g m^{-2} $year^{-1}$), and unfertilized control plots. In 2008, nutrient amendments began for BNCH, CDPT, CDR, KZA, LOOK, SEV, and SGS. Nutrient amendments to TREL began in 2009 and all plots had been under the same nutrient treatments for 10–11 years at the time of sampling.

2.2 | Sample collection

One hundred and eight samples were collected between 20 September and 13 December 2018. Because of the small experimental plot size, and the destructive nature of soil sampling, each plot-level sample (biological replicate) was limited to a composite of three, randomly selected 5 cm diameter × 15 cm deep cores to get good representation of the entire plot. The number of samples collected for each site was the same between treatment and control plots but differed among sites despite sampling all replicates at each site (either 3 or 4 biological replicates) as described in Table 1. In the field, roots and soil were separated using a 2 mm sieve, sealed in separate plastic bags, immediately placed on ice, and shipped overnight to Pacific Northwest National Laboratory. Upon arrival at the laboratory, root samples were gently washed with distilled water to remove soil particles and blotted dry to remove excess water. A subsample of roots was stored at −80°C for DNA isolation and the remaining roots were stored at 4°C for root staining for percentage mycorrhizal colonization.

Ecosystem and site properties (e.g., ecosystem type, location, elevation, mean annual precipitation, mean annual temperature, aridity index, N deposition, and soil pH, texture, and C, N, and P concentrations) were provided by Nutrient Network collaborators at https://nutnet.org/data (Table 1). Ecosystem type was defined based on the expertise of site leaders installing the NutNet sites using plant community, elevation, and climate data. Soil pH was measured by the method using 1:1 soil:water suspension. Soil texture (wt.% of sand, silt, and clay) was determined by the hydrometer method using a sodium-hexametaphosphate (HMP) solution as the dispersing agent (Ashworth et al., 2001). Soil total C and N % were measured by a Vario EL Cube CHNS elemental analyser from Elementar Americas, Inc. on a dry mass basis. Total soil P was measured by acid digestion in nitric acid and hydrofluoric acid, followed by ICP-OES. Total atmospheric N deposition ($kg\,N\,ha^{-1}\,year^{-1}$) was determined based on the modelled output of Ackerman et al. (2019).

2.3 | Root staining and assessment of arbuscular mycorrhizal colonization

A homogenized subsample of roots (0.1–0.2 g) was cut into 1-cm fragments and stained using the trypan blue staining procedure of Phillips and Hayman (1970). Roots were cleared in boiling 10% potassium hydroxide solution for 15–20 min, depending on thickness of roots. Once cleared, roots were rinsed with distilled water and then acidified by immersing in 2% hydrochloric acid for 1 h at room temperature. Roots were removed from hydrochloric acid solution and stained using 0.05% trypan blue solution. Stained roots were stored in lactoglycerol at 4°C until colonization assessment.

Colonization by AM fungi was assessed using the slide-intersect technique (McGonigle et al., 1990). Stained roots were mounted on microscope slides and observed at ×200 magnification using a compound microscope. For each sample, 100 intersects were analysed for presence/absence of AM fungal structures (i.e., hyphae, arbuscules, and vesicles). Total percentage mycorrhizal colonization of roots was calculated by dividing the number of intersections in which AM fungi was present by the total number of root intersections analysed and multiplied by 100.

2.4 | Root C, N and moisture content

Root samples were dried over a 2-day period at 45°C. Moisture content was calculated using the dry weight and predry weight. Dry roots were ground using Retsch MM400, and 3 mg per sample were weighed for the elemental analyser. C and N content is in weight percent (Bremner, 1996; Bremner & Mulvaney, 1983).

2.5 | Plant community composition and diversity

Plant community composition was measured as percent cover in each plot using a modified Daubenmire method by Nutrient Network site researchers (Daubenmire, 1959). Within each plot, a 1 m^2 subplot was selected and the percent cover was visually estimated to the nearest 1% for species rooted within the subplot. Plant community composition was measured at each site in the summer of 2017 and obtained from the 1 November 2020 release of the Nutrient Network Database. Since total cover can sum to >100% in a subplot using this method, each species cover was standardized to the maximum percent cover in each subplot. Plant community diversity was calculated using the Shannon Index (H′), plant richness refers to the number of species observed (S), and plant community evenness was calculated using Pielou's evenness [H′/log(S)]. Prior to analysis, plant community data were normalized using total sum scaling.

2.6 | AM fungal sequencing analysis

Frozen root samples were cut into 1-cm fragments and genomic DNA was isolated from 50 mg of frozen root tissue from each sample using the DNeasy PowerSoil kit (cat. no. 12888; Qiagen) following manufacturer's instructions with tissue lysing modification. Tissue lysing step was modified using the FastPrep Instrument (MP Biomedicals) with the following settings: speed: $6.0\,m\,s^{-1}$, MP: 24×2, and time 40 s (Cheeke et al., 2015). Isolated root DNA was stored at −80°C. Sequencing was done at Argonne National Laboratory on an Illumina MiSeq DNA sequencer using V3 2×300 reagent chemistry. Barcoded primers NS31 F and AML2 R were used, which target a ~550 bp region of the 18S rRNA gene that is highly specific for AM fungi taxa (Lee et al., 2008; Morgan & Egerton-Warburton, 2017; Simon et al., 1992). Sequences were demultiplexed and processed using the QIIME2 version 2020.2 implementation of DEMUX and DADA2 software packages respectively (Bolyen et al., 2019; Callahan et al., 2016; Hamday et al., 2008). We followed a similar protocol as Morgan and Egerton-Warburton (2017) to assign taxonomy for our study. Briefly, AM fungal taxonomy was assigned with the SILVA version 132 Qiime release using a pretrained Native Bayes classifier that was trained on the 18S SILVA 132 99% OTUs and 18S 99% all levels consensus taxonomy files with default settings (0.70 similarity; Bolyen et al., 2019; Quast et al., 2013). All sequences that were not identified as *Glomeromycota* were removed and not included in any downstream analyses. Taxonomy for amplicon sequence variants (ASVs) of interest (e.g., indicator ASVs) was confirmed with the Maarjam database (Öpik et al., 2010). AM fungal functional guilds were defined at the family level by the preferential distribution of hyphae into the root (rhizophilic; Glomeraceae, Claroideoglomeraceae, Paraglomeraceae), outside of the root (edaphophilic; Gigasporaceae, Diversisporaceae), or neither (ancestral; Archaeosporaceae, Ambisporaceae, Pacisporaceae, Acaulosporaceae; Weber et al., 2019). As is common with soil microbial data sets, 47.7% of the ASVs were unidentified at the family level and these unidentified reads were excluded from the functional guild analysis (Weber et al., 2019). Guild abundance was calculated from the relative abundance of associated sequences after total sum scaling normalization. Faith's phylogenetic diversity, Pielou's evenness, and observed richness values for each sample were calculated

after rarefying to 2000 sequences with QIIME2 (Bolyen et al., 2019). Sequences are available on the NCBI sequence read archive under accession number PRJNA725243.

2.7 | Statistical analysis

All statistical analyses were done using R version 4.0.3 statistical analysis software (R Core Team, 2019). Statistical analyses on the AM fungal community structure (i.e., the combined species composition, abundance, and diversity in a sample) were performed after total sum scaling normalization of the raw ASV table. The adonis function in the vegan package was used to run a PERMANOVA, which is robust to an unbalanced sample distribution, on a Bray–Curtis dissimilarity matrix to determine the response of AM fungal community structure to nutrient treatment, site and grassland ecosystem type (desert grassland, montane grassland, prairie, tallgrass prairie; Oksanen et al., 2017). Nutrient treatment, site, and ecosystem type were used as the predictive categorical variables and Bray–Curtis dissimilarity was included as the response variable in the PERMANOVA. The AM fungal community structure, identified via Bray–Curtis dissimilarity, was visualized with a two-dimensional NMDS ordination. Parameters were fit to the ordination using envfit in the vegan package (Oksanen et al., 2017). This means that the ordination axes values for each sample were correlated to plant community (e.g., root moisture) and environmental variables (e.g., pH) to identify if there was a significant relationship between AM fungal community structure and the variable of interest. Additional parameters used to explain characteristics of AM fungal community differences consisted of two main categories: environmental characteristics and plant community properties. Prior to inclusion in the final model, all variables were checked for multicollinearity and variables that were included in the final model had a Pearson's correlation coefficient <.7, with one exception. Soil pH and mean annual temperature (MAT) had a significant Pearson's correlation coefficient of .82 but were still included in the final visualization of the data due to their ubiquity in many environmental data sets. Soil percent N and C were strongly correlated with the aridity index (R^2 = .88 and .83, respectively) and were therefore not included in the final model. Environmental characteristics used in the final model were MAT, aridity index (AI), soil pH, and atmospheric N deposition (N_dep). Plant properties included in the analysis included total plant canopy cover (total cover), root moisture, root weight % N, root weight % C, and percent colonization of roots by AM fungi (AMF_colonization). We also calculated the plant NPP response ratio of each block-specific nutrient treatment as follows: nutrient response ratio = log(treatment NPP/control NPP) where NPP = sum of aboveground biomass and belowground NPP as described in (Fay et al., 2015). Aboveground biomass and belowground NPP were measured for the 2016–2017 growing season and come from (Keller et al., 2022a, 2022b). The NPP response ratio was also correlated with AM fungal community structure and colonization. A Pearson's correlation R^2 cutoff of .2 with ordination axes was used for including variables in visualization of envfit output.

An indicator species analysis was performed on the AM fungal ASV table using the indicspecies package (De Cáceres & Legendre, 2020). An indicator species analysis is a statistical technique that identifies taxa uniquely present in a specific group (McCune et al., 2002). Strong indicators were defined as having an indicator value >.70. The phylogenetic relationship of strong and significant AM fungal ASVs indicative of each ecosystem were compared and visualized as described in (Kasanke et al., 2019). Briefly, sequences for the indicator ASVs were aligned using MUSCLE (Edgar, 2004) and a tree was constructed using PhyML (Guindon et al., 2010) in SeaView (Gouy et al., 2010) and edited with iTOL (Letunic & Bork, 2016). Node confidence values (SH-aLRT) were calculated during tree construction (Anisimova et al., 2011). Due to the distinct phylogenetic relationship of the ancestral AM fungi relative to the other AM fungal guilds (see Weber et al., 2019 for guild descriptions), we were able to use the only ancestral species identified as an indicator species as the outgroup.

A three-factor ANOVA was performed to determine the effects of nutrient treatment, site, and ecosystem on AM fungal and plant diversity, evenness, percent root colonization, and fungal guild relative abundance (Kirk, 1995). In this model, nutrient treatment, site, and ecosystem type were predictive categorical variables and diversity, evenness, colonization, and fungal guild relative abundance were the response variables. Specific differences were identified with post-hoc Tukey tests. A p-value cutoff of .05 was used to determine statistical significance in all analyses. Values are reported as mean ± standard error unless specified otherwise.

3 | RESULTS

3.1 | Grassland ecosystem type best explains AM fungal and plant community variability

After sequence processing, 1,992,245 sequences were distributed among 2029 ASVs with a median of 16,461 sequences in each sample. Across all sites, the grassland ecosystem type (desert grassland, montane grassland, shortgrass prairie, tallgrass prairie) was the strongest explanatory variable influencing AM fungal colonization of plant roots (df = 3, F = 18.1, R^2 = .26, p < .001), with higher colonization rates in tallgrass prairie roots and lower colonization in desert grassland roots (Figure S1). Grassland ecosystem type also accounted for the most variability in AM fungal community structure (df = 3, F = 12.7, R^2 = .23, p < .001; Figure 2a) and explained the most variability in AM fungal diversity (df = 3, F = 10.0, R^2 = .21, p < .001), richness (df = 3, F = 12.3, R^2 = .24, p < .001) and evenness (df = 3, F = 2.8, R^2 = .07, p = .05) in roots (Table 2). In general, shortgrass prairie and desert grasslands trended lower than montane grasslands and tallgrass prairies on AM fungal diversity, richness, and evenness (Figure 3a–c). Grassland ecosystem impacts on AM fungal communities in roots reflected the patterns in plant communities (Table 2). Grassland ecosystem type best explained plant community structure differences (df = 3, F = 42.5, R^2 = .39, p = .001; Figure 2b),

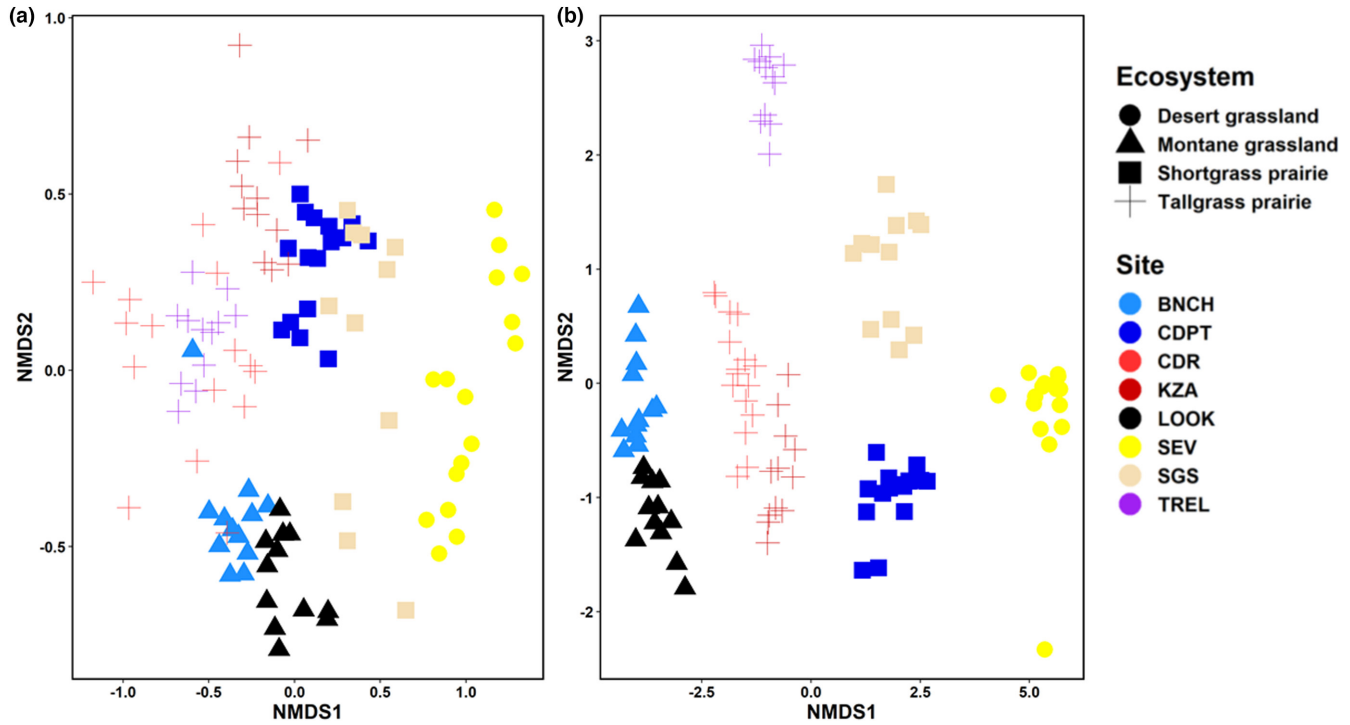

FIGURE 2 NMDS ordination showing bray–Curtis dissimilarity of (a) AM fungal communities (stress = 0.17) and (b) plant communities (stress = 0.04) from all treatment groups. Colours represent sites. Shapes represent ecosystem type. Although there is more overlap between AM fungal communities, both AM fungal and plant communities group primarily by ecosystem type. [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Statistics table of AM fungal and plant responses to ecosystem type, site-specific characteristics, and nutrient treatment, and AM fungal colonization, alpha diversity, evenness, and richness statistics come from an ANOVA test

	AM fungi						Plants					
	Ecosystem		Site		Nutrient		Ecosystem		Site		Nutrient	
	R^2	*p*-Value	R^2	*p*-Value	R^2	*p*-Value	R^2	*p*-Value	R^2	*p*-Value	R^2	*p*-Value
Colonization	**.26**	**<.001**	**.23**	**<.001**	.01	.604	NA					
Alpha diversity	**.21**	**<.001**	**.18**	**<.001**	.01	.637	**.44**	**<.001**	**.19**	**<.001**	**.03**	**.039**
Evenness	**.07**	**.047**	.04	.276	.04	.205	**.25**	**<.001**	.02	.380	.03	.102
Richness	**.24**	**<.001**	**.17**	**<.001**	.01	.725	**.22**	**<.001**	**.46**	**<.001**	.02	.095
Beta diversity	**.23**	**.001**	**.13**	**.001**	**.03**	**.001**	**.39**	**.001**	**.27**	**.001**	**.01**	**.013**

Note: Beta diversity statistics are from a PERMANOVA test. Numbers in bold indicate a *p*-value <.05.

plant diversity (df = 3, F = 45.2, R^2 = .44, p < .001) and evenness (df = 3, F = 17.7, R^2 = .25, p < .001) and explained a large amount of plant richness variability (df = 3, F = 24.4, R^2 = .22, p < .001). The trends in plant alpha diversity were largely explained by lower values overall in the desert ecosystem (Figure S2).

Grassland ecosystem type explained the most variability for each parameter (e.g., AM fungal colonization, diversity, composition), with one exception. Ecosystem type was second to site in explaining variability for overall plant richness, where both were significant, but site had a higher R^2. In addition, there was a significant site-specific secondary effect on AM fungal (df = 4, F = 5.5, R^2 = .13, p = .001) and plant (df = 4, F = 22.5, R^2 = .27, p = .001) community structure (Table 2). Site also had a strong secondary effect on AM fungal colonization (df = 4, F = 12.3, R^2 = .23, p < .001), diversity (df = 4, F = 6.3, R^2 = .18, p < .001), and richness (df = 4, F = 6.7, R^2 = .17, p < .001).

3.2 | Environmental drivers of AM fungal community composition

To identify the environmental factors that most strongly influenced AM fungal community structure, we analysed the continuous variables underlying the categorical ecosystem types. Together, environmental characteristics (aridity, mean annual temperature, pH, and atmospheric N deposition) underlying the ecosystem classification had the strongest correlation (R^2 = .43–.59) with the AM fungal community structure (Figure 4a; Table S1). Atmospheric

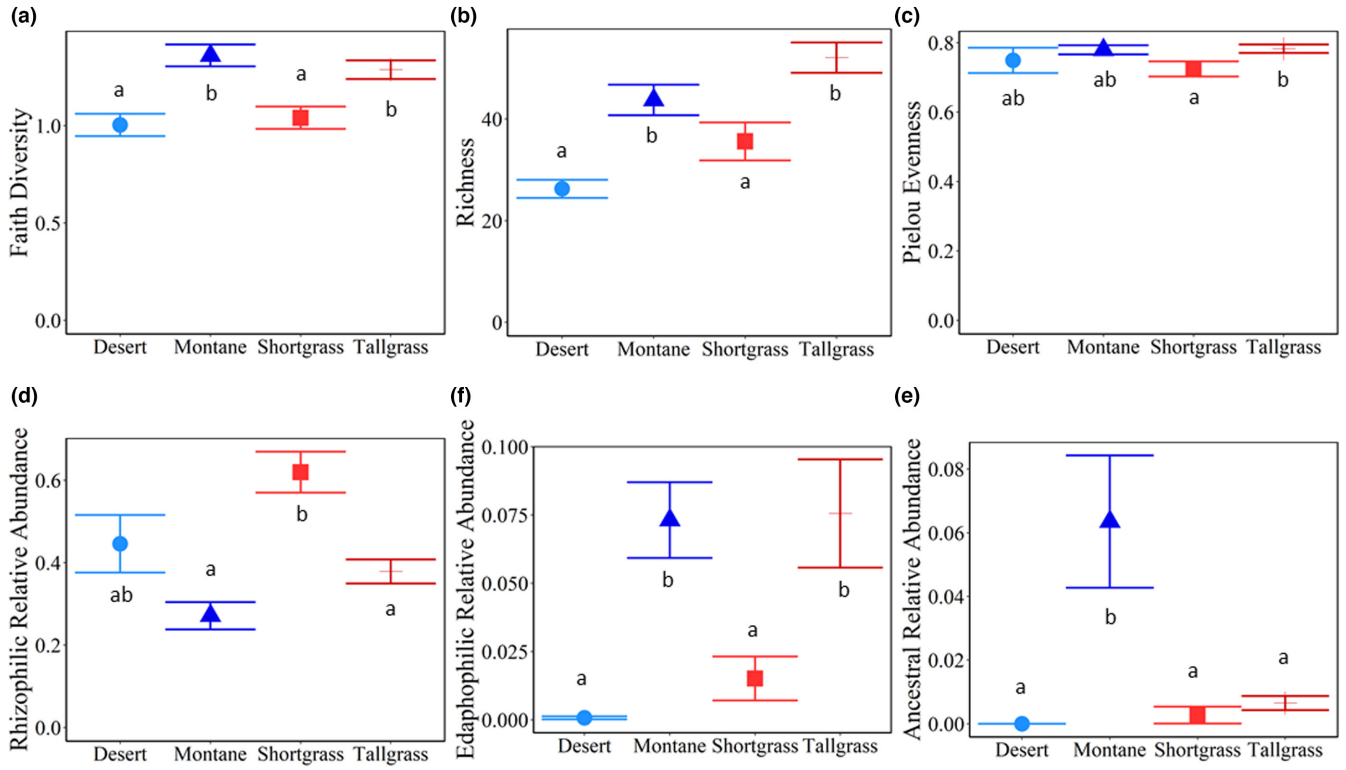

FIGURE 3 (a) Faith's phylogenetic diversity, (b) richness, and (c) Pielou's evenness of AM fungi in roots from each ecosystem type. Relative abundance of (d) rhizophilic, (e) edaphophilic, and (f) ancestral AM fungi in each ecosystem type. Error bars depict standard error of the means. [Colour figure can be viewed at wileyonlinelibrary.com]

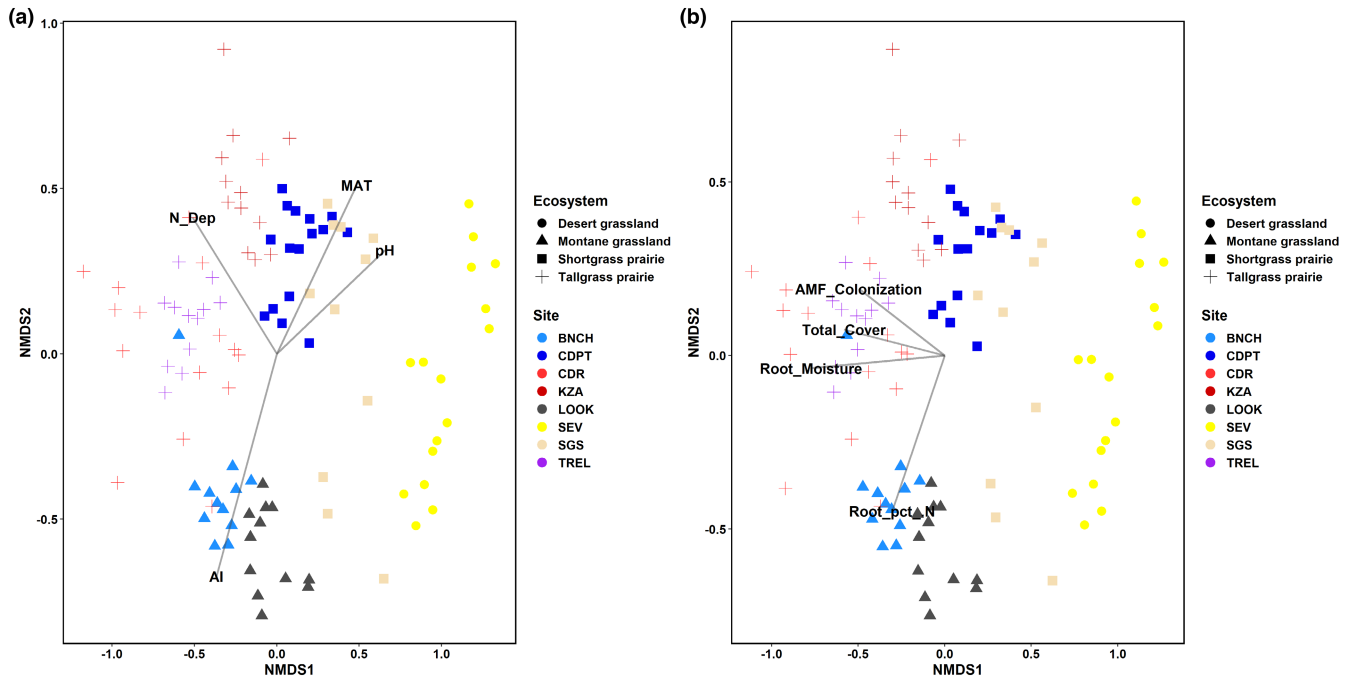

FIGURE 4 NMDS ordination of AM fungal community dissimilarity (bray-Curtis; stress = 0.17) showing the impact of site (a) and plant (b) characteristics on AM fungal community structure in roots. Vectors represent a measured variable with a Pearson's correlation coefficient of 0.2 or greater. AI, aridity index; AMF_colonization, percent of root colonized by AM fungi; MAT, mean annual temperature; N_Dep, atmospheric N deposition; pH, soil pH; root_moisture, root moisture weight percent; Root_pct_N, root N weight percent; total cover, total plant canopy cover. [Colour figure can be viewed at wileyonlinelibrary.com]

N deposition differed among grassland ecosystem types (df = 3, $F = 82.9$, $p < .001$) and was significantly higher in the tallgrass prairie ecosystems (Tukey, $p < .001$). Grassland ecosystem type also differed in MAT (df = 3, $F = 47.0$, $p < .001$), pH (df = 3, $F = 93.4$, $p < .001$), and aridity (df = 3, $F = 1819$, $p < .001$). Desert grasslands had the highest MAT (13.06°C) and montane the lowest (6.83°C); desert grasslands had the highest pH (7.3) and montane grasslands the lowest (5.5). Desert grasslands were the most arid and montane grasslands the least (Tukey, $p < .001$ all; Figure S3). Independently, root moisture, a plant characteristic, was most strongly correlated with the AM fungal community composition ($R^2 = .60$; Figure 4b) and differed between grassland ecosystem types (df = 3, $F = 73.95$, $p < .001$). Tallgrass prairies and montane grasslands had the highest root moisture content (Tukey, $p < .001$). Root nitrogen content (weight percent) also differed between ecosystem types (df = 3, $F = 29.8$, $p < .001$) and montane grassland roots also had the highest N content (Tukey, $p < .001$).

3.3 | Distribution of functional AM fungal guilds across four grassland ecosystem types

Due to the strong influence of grassland ecosystem type on other AM fungal parameters (e.g., diversity, community structure, root colonization), we also assessed the distribution of AM functional guilds across the four grassland ecosystem types (Figure 3d–f). The relative abundance of rhizophilic AM fungi differed between grassland ecosystem types (df = 3, $F = 10.5$, $p < .001$) (Figure 3d). Shortgrass prairies had a higher abundance of rhizophilic AM fungi than montane grasslands and tallgrass prairies (Tukey $p < .001$) and marginally more than desert grasslands (Tukey, $p = .063$). Edaphophilic was the second most abundant AM fungal guild (~5% relative abundance) with members also significantly influenced by grassland ecosystem type (df = 3, $F = 7.6$, $p < .001$). This was driven by montane grasslands and tallgrass prairies maintaining a higher relative abundance of edaphophilic AM fungi than desert grasslands and shortgrass prairie (Figure 3e). There was also a significant grassland ecosystem type effect on ancestral guild members (df = 3, $F = 12.9$, $p < .001$). Although ancestral AM fungal abundance was very low in most ecosystem types, they comprised a significantly higher proportion of montane grassland ecosystems (Tukey, $p < .001$; Figure 3f).

3.4 | Most grassland ecosystem indicator AM fungi are rhizophilic

We found phylogenetic differences between AM fungal ASVs that were indicative of each ecosystem type (Figure 5). Most of the AM fungal ASVs that were strong (IV >0.7) and significant ($p < .05$) indicators for the different grassland ecosystem types were members of the Glomeraceae family (rhizophilic), but they generally clustered together by ecosystem at finer taxonomic scales. However, montane grasslands, which had the most AM fungal indicators, did not fit this trend well. Two of the nine montane grassland indicators were non-rhizophilic; one was ancestral (Montane 9; *Archaeospora* sp. 99.6% identical) and another edaphophilic (Montane 5; *Scutellospora calospora* 98.8% identical). Two different montane grassland indicators, Montane 6 and 8, comprised the most deeply nested clade. There were no AM fungal ASVs that were indicative of tallgrass prairie ecosystems. Table S2 provides taxonomic details for all indicator AM fungal ASVs.

3.5 | Nutrient impacts on AM fungi in roots

We detected a significant site by nutrient treatment interaction on AM fungal community structure (df = 12, $F = 1.13$, $R^2 = .09$, $p < .001$). There was also a significant main effect of nutrient treatment on AM fungal community structure (df = 3, $F = 1.6$, $R^2 = .03$, $p = .004$), although it explained a small amount of the overall dissimilarity among AM fungal communities. We observed no significant nutrient treatment effect on AM fungal community alpha diversity indices (Faith_pd, richness, evenness) across sites. There was no detectable impact of nutrient treatment on colonization of roots by AM fungi (df = 3, $F = 0.6$, $p = .60$) across sites. There were no ASVs that were indicative of any nutrient treatment across grassland ecosystems and sites. We also investigated the differential AM fungal responses to site-specific nutrient limitation, by correlating AM fungal parameters with the site-specific NPP response ratio to N, P, and N + P. No significant trends were identified between the NPP response ratio and any AM fungal parameters (abundance, composition, diversity).

Rhizophilic AM fungi (those with preferential distribution of hyphae into the root) were by far the most abundant guild in all sites (~42% average relative abundance), and the abundance of rhizophilic fungi was not impacted by any of the nutrient treatments compared to the control plots (df = 3, $F = 0.7$, $p > .05$; Figure 6a). There was a higher relative abundance of edaphophilic AM fungi (those with preferential distribution of hyphae outside of the root) in plots amended with N (NP and N-only) than in plots amended with P-only (df = 3, $F = 3.9$, $p = .01$; Tukey, $p < .04$ both), but none of the nutrient additions altered the relative abundance of edaphophilic AM fungi compared to the control plots (Tukey, $p > .05$; Figure 6b). Ancestral AM fungi, the least abundant guild across all sites, were also impacted by nutrient additions (df = 3, $F = 4.9$, $p = .004$), but only in the N-only plots (Tukey, $p < .04$ all; Figure 6c) compared to the NP, P, and control plots. There was a significant grassland ecosystem type -by- nutrient interaction effect on the relative abundance of ancestral AM fungi (df = 9, $F = 4.1$, $p < .001$), in which ancestral AM fungal abundance was elevated in N-only plots in montane grassland and tallgrass prairie ecosystems (Figure S4).

The significant site-by-nutrient interaction on AM community structure indicates that AM fungal response to nutrient amendment differed among grassland sites. Therefore, the impact of nutrient amendments on AM fungal community structure and diversity were further investigated in each site independently.

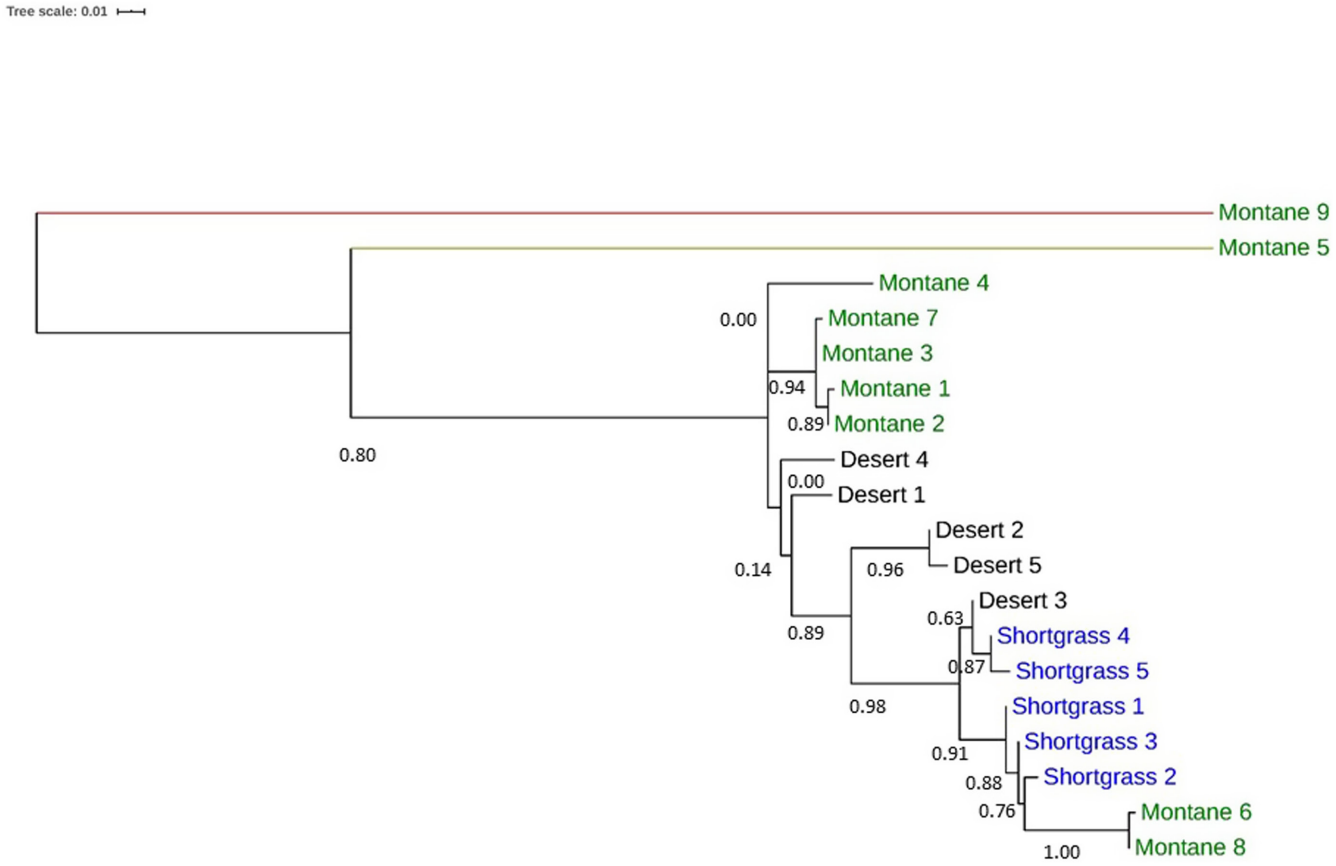

FIGURE 5 Phylogenetic relationship of all ASVs indicative of each grassland ecosystem type as determined by an indicator species analysis. The branches are coloured based on AM fungal functional guild classification with red representing ancestral AM fungi, yellow representing edaphophilic AM fungi, and black representing rhizophilic AM fungi. The labels are coloured by grassland ecosystem type. There were no indicator species for tallgrass prairie roots. Detailed taxonomic information for indicator ASVs are provided in Table S2. [Colour figure can be viewed at wileyonlinelibrary.com]

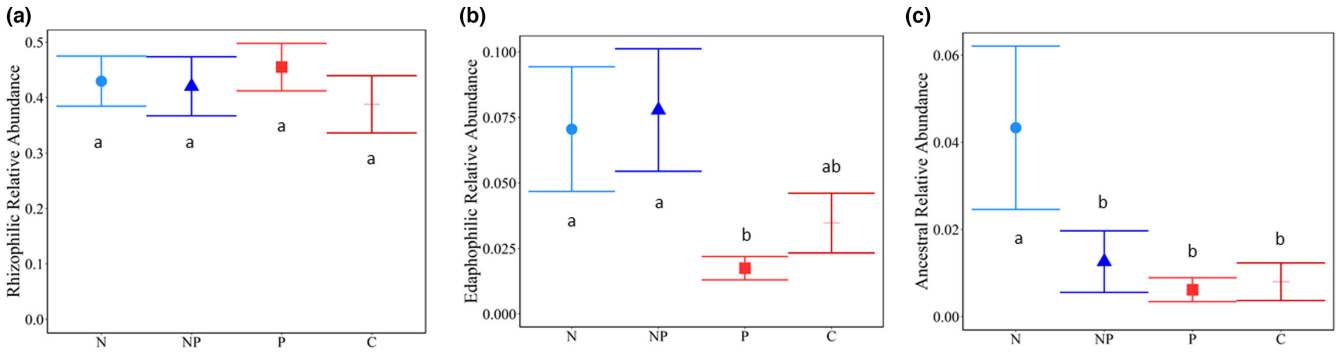

FIGURE 6 Nutrient impacts on the relative abundance (mean ± standard error) of (a) rhizophilic, (b) edaphophilic, and (c) ancestral AM fungi across all grassland ecosystems. Soils were amended with N only (N), N and P (NP), P only (P), and nonfertilized controls (C). [Colour figure can be viewed at wileyonlinelibrary.com]

Of the eight sites, only three showed significant nutrient treatment effects on AM fungal community structure including CDPT (df = 3, $F = 1.7$, $R^2 = .30$, $p = .03$), KZA (df = 3, $F = 1.6$, $R^2 = .38$, $p = .02$), and SGS (df = 3, $F = 2.1$, $R^2 = .52$; $p = .01$). While nutrient additions impacted AM fungal community structure in CDPT, KZA, and SGS, the impact was different in each site. In CDPT, there was a nutrient treatment impact on fungal richness (df = 3, $F = 3.9$, $p = .04$) attributed to a decrease in AM fungal richness in the NP plots compared to P-only plots (Tukey, $p = .05$). There were no other detectable differences in alpha diversity or AM fungal community evenness between nutrient treatments at CDPT. At KZA and SGS there was no difference in AM fungal alpha diversity, richness, or evenness between nutrient treatments, despite community compositional differences.

4 | DISCUSSION

This study represents an investigation of the long-term effects of environmental gradients on the distribution of AM fungal communities across North American grasslands. By taking a broad sampling of plant roots in different grassland ecosystems and a relatively new functional guild approach, we found that at the ecosystem scale, sustained nutrient additions did not strongly impact root AM fungal composition, diversity, or colonization across the grasslands tested.

4.1 | Moisture differentiates AM fungal guilds among ecosystems

Root moisture was the most strongly correlated variable with AM fungal community structure (Table S1). Similarly, the aridity index strongly correlated with AM fungal community structure. AM fungi have long been noted for increasing a plant's ability to access water (Allen, 2007; Mosse & Hayman, 1971; Ruth et al., 2011) and a recent estimate suggests 34.6% of the water transpired by a plant comes from AM fungi (Kakouridis et al., 2022). Our study supports evidence that AM fungal community composition is more strongly impacted by water availability than by N fertilization (Li et al., 2015). Increased AM fungal richness in the moist ecosystems (Figure 3) also fits a general trend of plant species richness increasing with moisture (Pausas & Austin, 2001). Although AM fungi are key players in plant-nutrient cycling, these results suggest that moisture may be more important in determining AM fungal distribution patterns than nutrient additions at the ecosystem scale.

Arbuscular mycorrhizal fungal community characteristics were most strongly influenced by grassland ecosystem type, where the distribution of AM fungal functional guild abundances was driven by environmental factors, such as moisture, and plant communities that are characteristic of each ecosystem. Rhizophilic AM fungi, noted for protecting their hosts from pathogens (Sikes et al., 2010), were the dominant guild in every grassland ecosystem sampled. This finding is consistent with studies in chaparral and coastal sage scrub ecosystems (Phillips et al., 2019; Weber et al., 2019). In our study, rhizophilic AM fungi were especially prominent in shortgrass and desert grassland ecosystems. Shortgrass and desert grasslands were the most arid ecosystems, and had the lowest values in root moisture, root N, and total plant cover (Figure 4). The potential pathogen protection offered by rhizophilic AM fungi may be important in these arid ecosystems possibly due to fewer plant hosts for pathogens to choose from (Bidzinski et al., 2016; Velásquez et al., 2018). Alternatively, rhizophilic AM fungi may be especially tolerant to harsh environmental conditions because proportionally more of their biomass is sequestered inside the plant root (Weber et al., 2019). Although the specific mechanisms for their dominance across ecosystems remains unidentified, rhizophilic AM fungi are relatively successful root colonizers overall, especially in drier ecosystems as demonstrated in our cross-ecosystem experiment.

In contrast, edaphophilic AM fungi with extensive extraradical hyphae networks, were most abundant in the moist environments. This finding supports the environmental modelling study that defined the AM fungal guilds (Weber et al., 2019) and concluded that edaphophilic AM fungi were the most impacted by reduced precipitation. In dry conditions, an extensive extraradical hyphal network may not be beneficial to the host or the fungus. Not only are there higher C costs to the plant for maintaining extraradical hyphae, there is also potential for increased moisture loss. The more C compounds a plant generates, the longer the plant's stomata need to be open, which could lead to moisture loss for the plant (Farooq et al., 2009). As a result, moisture in the arid environments may be insufficient for edaphophilic AM fungi to maintain extraradical hyphal networks.

The AM fungal communities strongly differ between the replicated montane grassland, tallgrass prairie, and shortgrass prairie ecosystems sampled (Figure 2). However, the differences in AM fungal guild abundances between deserts and the other ecosystems need to be examined further in future studies to identify idiosyncratic site differences or characteristics of desert grasslands more generally. While all of the sites we sampled from had at least 3–4 replicates per treatment, one limitation of our study is that we were only able to sample one desert ecosystem site. Desert ecosystems are underrepresented in ecological experiments and are important to include in cross site studies. We also note that the AM functional guild framework was developed based on studies where AM fungi were cultured, and their relative biomass distributions were reported (Weber et al., 2019). As reported by Weber et al. (2019), here we apply this framework in the field context; however, because most AM fungal species are not yet cultured, these results should be interpreted with this caveat in mind.

4.2 | Impact of pH, aridity, and atmospheric N deposition on AM fungi

As is commonly found, soil pH correlated with AM fungal community structure (Bainard et al., 2014; Hazard et al., 2013; Melo et al., 2019). In our study, the impact of pH on AM fungal community structure was inversely related to the impact of aridity on AM fungal community structure with more arid soils also having a higher soil pH (Figure 4a). Therefore, it is possible that the changes in guild abundances can also be attributed to pH differences among ecosystems. These findings have important implications for how fungal ecology may shift in response to changing environmental conditions. Global circulation models predict increases in extreme regional weather events, including precipitation and drought (IPCC, 2022). Because higher moisture reduces soil pH by promoting weathering and leaching of base cations, it is likely shifts in precipitation will alter aridity and soil pH, resulting in changes in plant community composition, as well as direct effects on structural and functional AM fungal communities.

In our study, atmospheric N deposition did not co-occur with other environmental variables in ordination space and strongly

correlated with AM fungal community structure. Sites with higher atmospheric N deposition rates also had higher root colonization (Figure 4). This aligns with documented impacts of N deposition on AM fungi in chaparral ecosystems, where increases in richness and relative abundance were positively correlated with nitrate deposition and negatively correlated with ammonium deposition (Phillips et al., 2019). Mean annual temperature was highly correlated with the aridity index, which was decoupled from atmospheric N deposition impacts on AM fungal community structure. Although we were unable to generate N speciation data for the atmospheric N deposited at each site, the positive relationship between N deposition and percent colonization indicated that nitrate may be the dominant component deposited at these sites. This agrees with data from the National Atmospheric Deposition Programme (NADP) which shows that although trends may be shifting, there was more nitrate than ammonia deposited across the regions these sites are located in the year we sampled (http://nadp.slh.wisc.edu/data/). Together, these findings suggest that correlating AM fungal percent root colonization with atmospheric N deposition may be a useful bioindicator in determining the dominant form of atmospheric N deposited in comparative ecosystem studies.

4.3 | Minimal impact of long-term fertilization on grassland AM fungi

The results of our study are in contrast with conceptual models that predict a negative response of AM fungi to soil nutrient additions (Ma et al., 2020). While many studies predict decreased AM fungal diversity and/or colonization in roots under fertilized versus unfertilized plots (Jach-Smith & Jackson, 2018; Ma et al., 2020; Treseder, 2004), they are often based on greenhouse studies or relatively short field studies. Although, in our long-term field-based study we found no detectable increase in total soil N after 10 years of fertilization (Keller et al., 2022a, 2022b; Table S3), N amendments did elevate inorganic N concentrations in soils (J. DeLancey, personal communication, 11 November 2022), which should have increased N availability to AM fungi. Nevertheless, we did not detect a consistent long-term effect of added N on root-associated AM fungal percent colonization or alpha diversity in any of the sites.

Unlike previous findings of rhizophilic AM fungi decreasing after N addition (Han et al., 2020), we found no consistent taxonomic or guild-specific response of root-associated AM fungi to nutrient additions across sites or ecosystems. We predicted a decrease of edaphophilic AM fungi and an increase of rhizophilic AM fungi in fertilized plots, but this was not supported. Instead, only relative abundances of the ancestral guild differed between nutrient-amended and control plots. In plots amended with N alone, ancestral AM fungal abundance was elevated in montane grasslands and tallgrass prairie ecosystems. Ancestral AM fungi may have the lowest C demand from the plant host due to the relatively low amount of both intraradical and extraradical hyphae (Weber et al., 2019). However, no treatment effects were detected for N + P or P fertilization regimes. Even when nutrient limitation of each site was accounted for by the NPP response ratio, no significant trends were identified between nutrient amendments and percent AM fungal colonization.

The lack of a strong nutrient treatment effect on AM fungi was unexpected based on the dominant conceptual model for interactions between AM fungi and plants. Previous studies reported that nutrient additions were associated with lower levels of AM fungal colonization in roots (Johnson et al., 2003) and lower relative abundance of AM fungi in both soil and roots (Egerton-Warburton et al., 2007; Leff et al., 2015). Although a significant overall decrease of AM fungal abundance was observed with N and P amendments in a previous NutNet study (Leff et al., 2015), explanatory power was low ($R^2 = .003$), and site-specific responses were highly variable with AM fungal abundance, increasing >100% from the controls in some plots.

The importance of site specific properties over nutrient additions on AM fungal abundance is also reinforced in a recent study using structural equation modelling (Lekberg et al., 2021). AM fungi are intimately associated with their plant hosts, and Prober et al. (2015) demonstrated that across NutNet sites plant beta diversity correlates with bacterial and fungal beta diversity, which is further supported by Leff et al. (2015), who demonstrated that plant communities which respond most strongly to nutrient additions also show strong microbial responses ($R^2 = .44$). In our study, after 10 years of fertilization, plant community characteristics (diversity, composition) were also minimally affected by nutrient amendments across these sites (Table 2). Therefore, the dominant environmental controls on plant community composition, not just above-ground biomass, may also strongly influence AM fungal communities. Our study enhances our understanding of the niche partitioning of functionally different AM fungi and provides insights into their different in situ lifestyle strategies, emphasizing the importance of long-term field scale research projects to identify important biological responses not otherwise achievable.

5 | CONCLUSION

Our study, among others (Phillips et al., 2019; Weber et al., 2019), demonstrates that fungal functional guilds can provide a strong framework for assessing the relative impacts of host-selection and environmental tolerances across ecosystems. Despite a decade of nutrient additions, we found that AM fungal diversity, community structure, and functional guild abundances were most influenced by the grassland ecosystem type they inhabited. Ecosystem differences in AM fungi were related to differences in moisture availability, a parameter easily measured and incorporated into predictive models. Thus, even though AM fungi are key players in plant-nutrient cycling, soil moisture may be more important in determining AM fungal distribution patterns than fertilization, especially as global temperatures rise and water stress becomes more prevalent.

AUTHOR CONTRIBUTIONS

Kirsten S. Hofmockel, Sarah E. Hobbie and Tanya E. Cheeke planned and designed the research. Trinidad Alfaro, Qian Zhao and Tanya E. Cheeke performed experiments, Christopher A. Walter conducted fieldwork, Christopher P. Kasanke, Trinidad Alfaro and Qian Zhao analysed data. Christopher P. Kasanke drafted the manuscript and all authors provided significant revisions.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data necessary to reproduce these results have been made available at the following locations: Plant and Environmental data are available upon request from the Nutrient Network database at https://nutnet.org/data. AM fungal sequences have been made available on the NCBI sequence read archive under accession number PRJNA725243.

BENEFIT-SHARING STATEMENT

The nutrient network consists of a network of long-term grassland field sites experience the same nutrient and herbivory treatments. This research team benefited from and contributed to the efforts of this program.

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